ISSN 00 22-09 81
JEMBAM 46(2,3) 127–298 (1980)
0, Volume 46, Number 2,3, pp. 127–298 Published 16 July 1980

JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

LIBRARY
AUG 11 1980

ELSEVIER/NORTH-HOLLAND

JOURNAL OF EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

Harold Barnes, Founder Editor

SCOPE OF THE JOURNAL

Papers on all aspects of marine ecology – floristic or faunistic, littoral, inshore, offshore, and deep sea – will be acceptable. The journal is particularly intended, however, to be a forum for work on the physiology, biochemistry, and behaviour of organisms in relation to their ecology; experimental work on eco-systems and on individual organisms in relation to their ecology will be especially welcome. Although this kind of work will be stressed, papers of a descriptive character – particularly if they aim at elucidating or illuminating general ecological problems and principles – will be considered for inclusion. Articles on new techniques and methods will, if their importance warrants it, be published, as will those concerned with 'theoretical' ecology. Every effort will be made to expedite publication.

Managing editors

Margaret Barnes, The Dunstaffnage Marine Research Laboratory, Oban, Argyll PA34 4AD, Scotland

F. John Vernberg, Belle W. Baruch Institute for Marine Biology and Coastal Research, University of South Carolina, Columbia, SC 29208, U.S.A.

Associate editor

Robin N. Gibson, The Dunstaffnage Marine Research Laboratory, Oban, Argyll PA34 4AD, Scotland

Editorial board

D. T. Anderson, Sydney, Australia
A. C. Brown, Cape Town, South Africa
L. Cabioch, Roscoff, France
A. Jensen, Trondheim, Norway
P. Moreira, Sao Paulo, Brazil
F. D. Por, Jerusalem, Israel
G. Stephens, Irvine, Calif., U.S.A.
J. Yamada, Hakodate, Japan

Instructions to authors: see inside back cover.

Subscription information

The subscription price of a volume is Dfl. 132.– or U.S. $ 67.75, including postage.

Subscriptions should be sent to the publishers, **Elsevier/North-Holland Biomedical Press, Journal Division, P.O. Box 211, 1000 AE Amsterdam, The Netherlands.**

Claims for missing numbers should be made within two months, following the date of publication. The publishers only expect to supply missing numbers free when losses have been sustained in transit and if the reserve stock will permit.

© Elsevier/North-Holland Biomedical Press, 1980

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior permission of the copyright owner.

Submission to this journal of a paper entails the author's irrevocable and exclusive authorization of the publisher to collect any sums or considerations for copying or reproduction payable by third parties (as mentioned in article 17 paragraph 2 of the Dutch Copyright Act of 1912 and in the Royal Decree of June 20, 1974 (S. 351) pursuant to article 16b of the Dutch Copyright Act of 1912) and/or to act in or out of Court in connection therewith.

Library of Congress Catalog Card Number 68–26535

Printed in Belgium

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 127–135
© Elsevier/North-Holland Biomedical Press

COMPARATIVE STUDIES OF MUCOPOLYSACCHARIDES FROM MARINE ANIMALS. I. *RAJA EGLANTERIA* Bosc.

B. PATEL[1]

Stevens Institute of Technology, Department of Chemistry and Chemical Engineering, Hoboken, NJ 07030, U.S.A.

J. EHRLICH

College of Medicine and Dentistry of New Jersey, Newark, NJ 07103, U.S.A.

and

S. S. STIVALA and N. K. SINGH[2]

Stevens Institute of Technology, Department of Chemistry and Chemical Engineering, Hoboken, NJ 07030, U.S.A.

Abstract: A mucopolysaccharide (MPS) was extracted from the pectoral fins of the skate, *Raja eglanteria* Bosc. The purified MPS had an anticoagulant activity of 28 IU/mg and an optical rotation value, $[\alpha]_D^{25}$, of $-9°$. The MPS was analyzed for uronic acid, hexosamine, *N*-sulfate, *O*-sulfate, and neutral sugar. This analysis suggests that this MPS is an over-sulfated chondroitin sulfate, the electrophoretic pattern and infra-red spectrum of which are similar to chondroitin sulfate.

INTRODUCTION

Comparative studies have been made on the mucopolysaccharides (MPS) of the connective tissues of various animals, including invertebrates and lower vertebrates. It has been found that the mucopolysaccharide composition and structure are quite different from that of the higher animals. In the cyclostomes, new types of hyaluronic acid as well as dermatan polysulfate were isolated by Anno *et al.* (1971). Hagfish skin yielded a dermatan sulfate which contained a trisulfated disaccharide residue (Seno *et al.*, 1972) and from the hagfish notochord chondroitin sulfate-H was isolated (Anno *et al.*, 1971). The chondroitin sulfate isolated from shark cartilage by Nakanishi *et al.* (1956) and Soda *et al.* (1940), contained an unusually high sulfate content. Partially over-sulfated chondroitin sulfate C preparation from shark cartilage has been reported (Mathews *et al.*, 1962). From a comparative point of view, it is of interest to compare the mucopolysaccharides of the amphioxus (*Branchiostoma belcherii*) which belongs to the Cephalochorda, the highest invertebrates. The com-

Present addresses:
[1] Roy Weston, Inc., West Chester, PA 19380, U.S.A.
[2] Penick Corporation, Newark, NJ, 07114, U.S.A.

position of mucopolysaccharides from amphioxus notochord differs from that of hagfish or lamprey and the content from the amphioxus skin is more similar to that of vertebrates than that of cephalopods (Anno & Kawai, 1975).

Heparin is a mucopolysaccharide composed essentially of partially sulfated units of α-D-glucuronic acid and 2-amino-2-deoxy-α-D-glucose joined by $1 \rightarrow 4$ bonds. It is mainly noted for its anticoagulant activity in the treatment of various cardiovascular diseases, but it is also an antilipaemic agent. Heparin originates in the mast cells, inter alia of connective tissue, and is found in beef, hog and dog livers, beef muscle, adipose tissue and spleen, hog mucin and alimentary canal, skin of rats, fish plasma, sea clams, and the scales of carp. The chemistry and pharmacology of heparin was recently reviewed by Ehrlich & Stivala (1973). Heparinoid activity has also been reported in chondroitin sulfate C (Nagasawa *et al.*, 1972). The present investigation concerns itself mainly with the extraction and physico-chemical characterization of heparin and heparin-like substances from marine animals. This paper describes the isolation and preliminary characterization of mucopolysaccharides from the tissue of the skate, *Raja eglanteria* Bosc.

Materials and Methods

The pectoral fins of the skate, *R. eglanteria*, supplied by the Osborn Laboratories of the New York Aquarium were separated, blended, and stored at −20 °C until the isolation process was initiated. Batches of 1000 g of tissue were thawed and de-fatted with 1 l of acetone, filtered and further de-fatted with 1 l of petroleum ether. The tissues were then air-dried at room temperature and vacuum dried at 45 °C, overnight.

Extraction

Dry de-fatted tissues (100 g) were suspended in 1 l of 0.1 M Tris–HCl 0.1 M $CaCl_2$, pH 8.0, containing 4 ml of toluene. 300 mg of papain were added and the mixture stored at room temperature for 24 h, followed by an additional 200 mg of papain for the same period of time. After digestion, solid NaCl was added to make a 1-M concentration. Solids were removed immediately by vacuum filtration and then were discarded. The filtrate was centrifuged at room temperature, to remove all traces of sediments (20 min at 5800 *g* in a Sorvall RC2-B centrifuge).

Treatment with cetyl pyridinium chloride (CPC)

The supernatant was diluted to 0.8 M NaCl concentration, warmed to 40 °C followed by slow addition of a CPC solution (3 g/100 ml of 0.8 M NaCl) with stirring until precipitate formation was completed. The CPC was used to complex the MPS for their separation from other solutes, e.g. proteins, neutral polysacchari-

des. The suspension was incubated at 37 °C overnight, and was then centrifuged at the same temperature at 5800 *g*. The supernatant was discarded and an appropriate amount of warm (40 °C) 2 M NaCl solution was added to the solid CPC–MPS complex to effect separation of the MPS from the complex. After filtering the suspension the filtrate was mixed with 2 vols of ethanol, 1 vol of water, stirred and finally an additional 2 vols of 95% ethanol, and stored at 4 °C overnight to re-precipitate the MPS. The precipitated MPS were centrifuged, re-dissolved in distilled water and freeze-dried.

FRACTIONATION

The freeze-dried MPS were dissolved in a small volume of water and applied to an anion-exchange column (Dowex-1 × 10 Cl^{-1} 200–400 mesh, size 2 × 25 cm), and then eluted stepwise with 250 ml of 0.5 M, 1.0 M, 2.0 M, and 4.0 M NaCl. For each step, 50 fractions of 5 ml were collected. The contents of each tube were assayed for hexuronic acid by the method of Bitter & Muir (1962) to determine which contained mucopolysaccharides. The fractions containing mucopolysaccharides were dialyzed using Spectrapor membrane tubing (m.w. cut-off 3000) against distilled water, and lyophilized (see flow diagram, Fig. 1).

ANALYTICAL METHODS

Uronic acid was measured by the modification of Bitter & Muir (1962) of the carbazole method of Dische (1947), and by the orcinol method of Mejbaum (1939). Glucuronic acid was the standard used in both cases. Hexosamine was determined by the method of Boas (1953), after hydrolysis with 4 N HCl for 15 h at 100 °C. Total sulfate was determined by the $BaCl_2$-gelatin method of Dodgson (1961) and Dodgson & Price (1962) after hydrolysis of the MPS with 1 M HCl at 100 °C in a closed tube for 15 h. *N*-sulfate was estimated by the method of Lagunoff & Warren (1962). Protein was assayed by the method of Bradford (1976) using bovine serum albumin as a standard, and neutral sugar was determined by the anthrone assay of Spiro (1966). Purity of the sample was determined by gel-filtration using Sephadex G200, and eluants were assayed for hexuronic acid by the method of Bitter & Muir (1962) using 0.025 M NaCl for the elution.

ELECTROPHORESIS

Electrophoresis was carried out in a Gelman electrophoresis apparatus, on Agarose gel plates (Agarose Universal Film, Fisher Scientific Co.), in acetate buffer pH 3.0, for 15–20 min, at a constant voltage of 300 V. The following reference standards were run simultaneously: chondroitin sulfate (99% pure ICN Pharmaceutical Co.), heparin (Organon), heparitin sulfate, and kerato-sulfate (from Drs. M. B. Mathews and J. A. Cifonelli, Department of Pediatrics, School of Medicine,

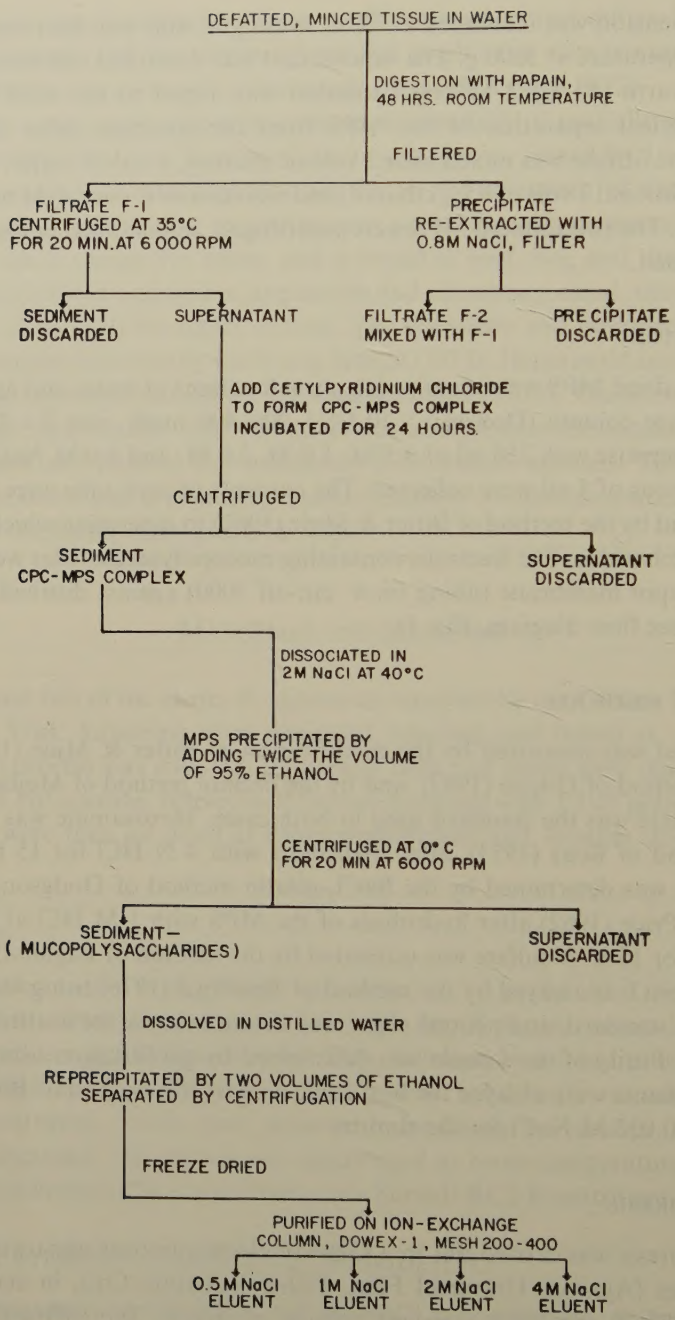

DEFATTED, MINCED TISSUE IN WATER
DIGESTION WITH PAPAIN, 48 HRS. ROOM TEMPERATURE
FILTERED
FILTRATE F-1 CENTRIFUGED AT 35°C FOR 20 MIN. AT 6 000 RPM
PRECIPITATE RE-EXTRACTED WITH 0.8M NaCl, FILTER
SEDIMENT DISCARDED
SUPERNATANT
FILTRATE F-2 MIXED WITH F-1
PRECIPITATE DISCARDED
ADD CETYLPYRIDINIUM CHLORIDE TO FORM CPC-MPS COMPLEX INCUBATED FOR 24 HOURS.
CENTRIFUGED
SEDIMENT CPC-MPS COMPLEX
SUPERNATANT DISCARDED
DISSOCIATED IN 2M NaCl AT 40°C
MPS PRECIPITATED BY ADDING TWICE THE VOLUME OF 95% ETHANOL
CENTRIFUGED AT 0° C FOR 20 MIN AT 6000 RPM
SEDIMENT— (MUCOPOLYSACCHARIDES)
SUPERNATANT DISCARDED
DISSOLVED IN DISTILLED WATER
REPRECIPITATED BY TWO VOLUMES OF ETHANOL SEPARATED BY CENTRIFUGATION
FREEZE DRIED
PURIFIED ON ION-EXCHANGE COLUMN, DOWEX-1, MESH 200-400
0.5M NaCl ELUENT
1M NaCl ELUENT
2M NaCl ELUENT
4M NaCl ELUENT

Fig. 1. Flow diagram for the extraction and purification of MPS.

University of Chicago). The MPS were visualized by staining the strips in 1% toluidine blue and excess stain was removed by washing with water.

INFRARED ANALYSIS (IR)

Infrared spectra were obtained from samples in potassium bromide pellets on a Perkin-Elmer spectrophotometer.

OPTICAL ROTATION

Optical rotation was measured with a Rudolph Polarimeter at 25 °C.

ANTICOAGULANT ACTIVITY

Anticoagulant activity was measured by the kaolin-activated partial thromboplastin time test (Highland, Division Travenol Laboratories, Inc.) with a fibrometer.

Results and Discussion

The elution pattern of mucopolysaccharides from the Dowex Cl^- anion-exchange column showed a single assymmetrical peak (Fig. 2) when 2 M NaCl was the

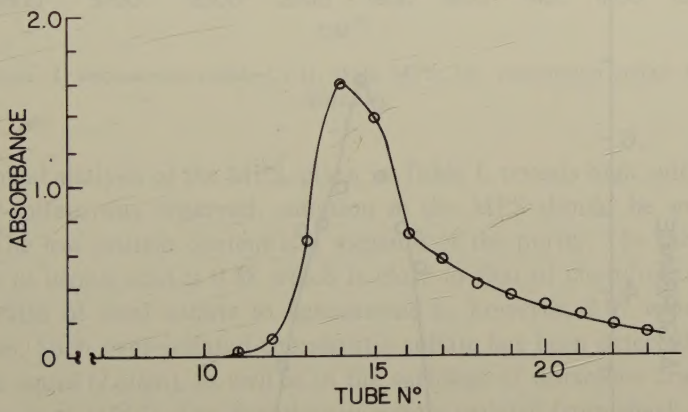


Fig. 2. Anion exchange chromatography of skate MPS with 2.0 M NaCl eluant.

eluant. Agarose gel electrophoresis showed one band of similar mobility to chondroitin sulfate (Fig. 3). The gel filtration of the MPS also suggests a single component (Fig. 4).

The infrared spectrum of the MPS is shown in Fig. 5 and shows a strong peak at 1240 cm^{-1} suggesting the presence of ester sulfate groups. The absorption band for the carboxylic group at 1615 cm^{-1} and for acetyl amino groups at 1375 cm^{-1}

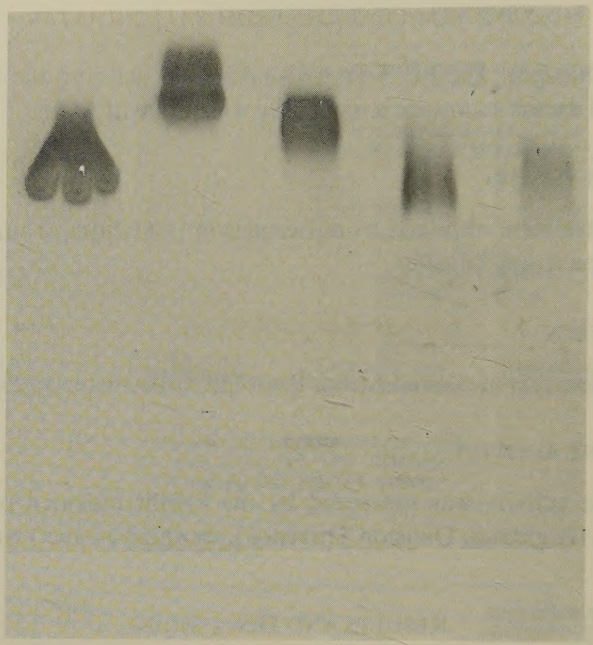


Fig. 3. Electrophoretic patterns on Agarose strip, reading from left to right: skate MPS, heparin, chondroitin sulfate, heparitin sulfate, keratosulfate.

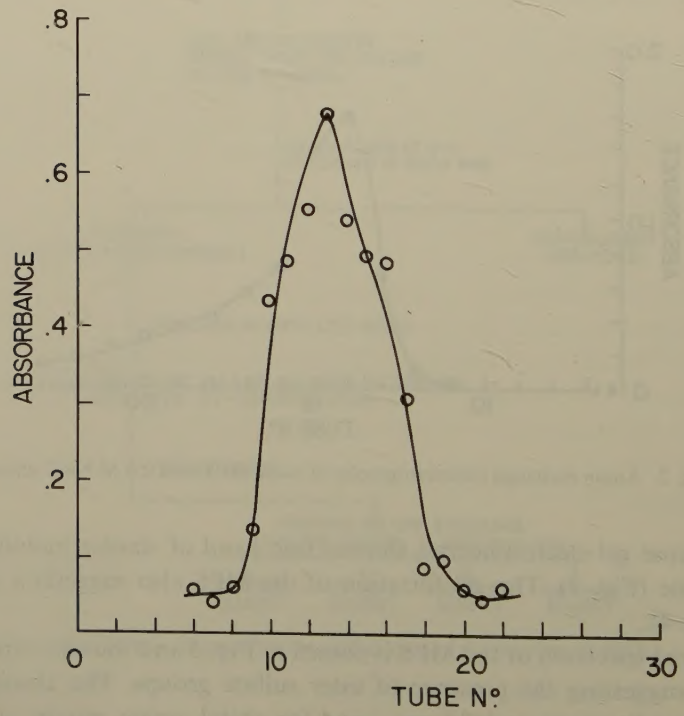


Fig. 4. Gel filtration of skate MPS on Sephadex using 0.025 M NaCl eluant.

were observed as reported by Rahemtulla & Lovtrup (1974) on acid mucopolysaccharides from Cestoda. Whether the sulfate linkages are at C-4 or C-6 cannot be distinguished from the infrared spectrum. The optical rotation, $[\alpha]_D^{25°} = -9°$, is, however, similar to that of chondroitin sulfate-C as reported by Roden *et al.* (1972).

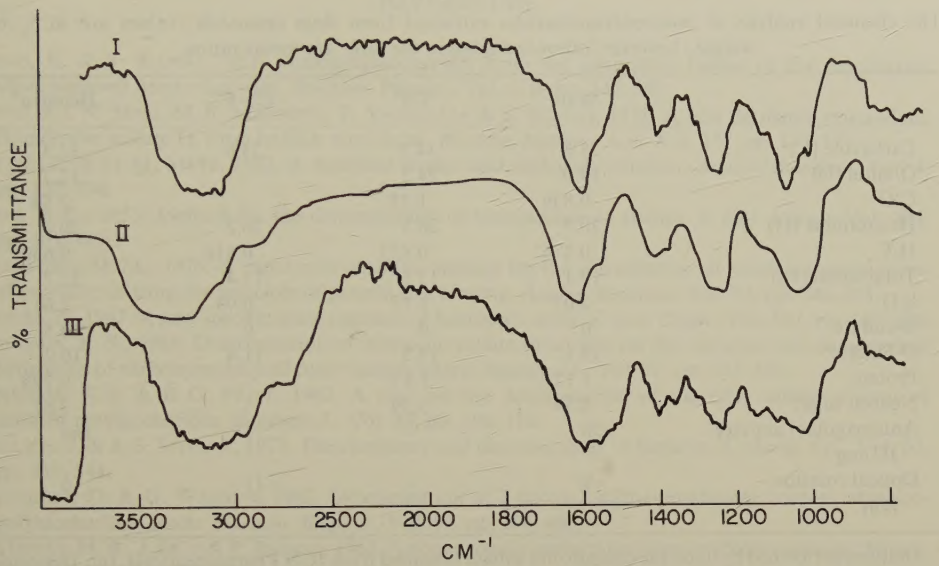


Fig. 5. IR spectra: I, chondroitin sulfate-C; II, skate MPS; III, chondroitin sulfate (99% Pharmaceuticals).

The chemical analysis of the MPS, given in Table I, reveals high sulfate content. Since no *N*-sulfate was observed, sulfation in the MPS should be solely due to *O*-sulfate. The low protein content is a measure of the purity. The molar ratio of hexosamine to uronic acid is 0.88 which is close to that of chondroitin sulfate-C; the molar ratio of total sulfate to hexosamine is, however, 2.07 which indicates oversulfation. Such over-sulfated chondroitin sulfate has been detected in the cartilage of the squid (*Loligo*), as well as in the cartilage of horseshoe crab (*Limulus*) by Mathews *et al.* (1962). The chondroitin sulfate isolated from shark cartilage by Nakanishi *et al.* (1956), Soda *et al.* (1940), and Utimaru (1943) has an infrared spectrum identical with that of chondroitin sulfate-C; the shark chondroitin sulfate has, however, an unusually high sulfate content (S/H = 1.3). Since these two polysaccharides also differ as acceptors in enzymatic sulfation, Suzuki & Strominger (1960) designated the chondroitin sulfate from shark cartilage as chondroitin sulfate-D. The MPS isolated by us from the skate is also high sulfated, and even although it has a low optical rotation ($[\alpha]_D^{25°} = -9°$), close to that of chondroitin sulfate-C, and other similarities in chemical analyses, the degree of sulfation is much

higher (S/H = 2.07), and this even exceeds that obtained by Suzuki & Strominger (1960) for chondroitin sulfate-D. Furthermore, the carbazole/orcinol (C/O) ratio in the skate is <1, which indicates a high iduronic acid content, compared with the C/O ratio for chondroitin sulfate.

TABLE I

The chemical analysis of mucopolysaccharides extracted from *Raja eglanteria*; values are in % by weight; however, ratios indicated in the table are molar ratios.

	Skate	CS[a]	CS–C[b]	Heparin[d]
Carbazole (C)	25.6	34.3	34.6	32.3
Orcinol (O)	29.9	24.2	–	12.7
C/O	0.856	1.33	–	2.54
Hexosamine (H)	20.9	26.5	26.2	20.3
H/C	0.876	0.833	0.816	0.680
Total sulfate (S)	19.1	13.5	11.4	23.7
S/H	2.07	1.15	0.98	2.61
N-sulfate	0	0	0	13.5
O-Sulfate[c]	19.1	13.5	11.4	10.2
Protein	1.12	1.81	–	0.799
Neutral sugar	4.54	5.39	–	3.67
Anticoagulant activity IU/mg	28	–	–	164
Optical rotation $[\alpha]_D^{25}$	−9°	–	−11°	0

[a] Analyses performed by us on the chondroitin sulfate obtained from ICN Pharmaceuticals, Inc. (reported to be 99%).
[b] Data for chondroitin sulfate-C taken from Roden *et al.* (1972).
[c] Obtained by subtraction of *N*-sulfate from total sulfate.
[d] Bovine lung, Organon, Inc.

It is apparent that the mucopolysaccharide extracted from the skate is a form of chondroitin sulfate similar to that extracted by Nakanishi *et al.* (1956) from shark tissue. As the total sulfate values in the skate mucopolysaccharides are higher than those from the shark, it would appear that this is a distinguishing feature of this material. Furthermore, the anticoagulant activity measured was 28 IU/mg, which reflects a higher sulfate content than that of chondroitin sulfate-C, 9.4 IU/mg given by Nagasawa *et al.* (1972). Thus it appears that the mucopolysaccharide extracted from the skate is a form of chondroitin sulfate, similar to but not identical with, most other chondroitin sulfates. Accordingly, we designate this as chondroitin sulfate-S indicating the chondroitin sulfate extracted from the skate.

Subsequent papers will describe mucopolysaccharide from crustaceans and molluscs.

Acknowledgement

This work is a result of research sponsored, in part, by NOAA, Office of Sea Grant, Department of Commerce, under grant number 04-7-158-44042.

References

Anno, K. & Y. Kawai, 1975. Mucopolysaccharides from the connective tissues of the amphioxus, *Branchiostoma belcherii*. *Comp. Biochem. Physiol.*, Vol. 52B, pp. 547–549.

Anno, K., N. Seno, M. B. Mathews, T. Yamagata & S. Suzuki, 1971. A new dermatan polysulfate, chondroitin sulfate H, from hagfish notochord. *Biochim. biophys. Acta*, Vol. 237, pp. 173–177.

Bitter, T. & H. M. Muir, 1962. A modified uronic acid carbazole reaction. *Analyt. Biochem.*, Vol. 4, pp. 330–334.

Boas, N. F., 1953. Method for the determination of hexosamines in tissues. *J. biol. Chem.*, Vol. 204, pp. 553–563.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. *Analyt. Biochem.*, Vol. 72, pp. 248–254.

Dische, Z., 1947. A new specific color reaction of hexuronic acids. *J. biol. Chem.*, Vol. 167, pp. 189–198.

Dodgson, K. S., 1961. Determination of inorganic sulfate in studies on the enzymic and non-enzymic hydrolysis of carbohydrates and other sulfate esters. *Biochem. J.*, Vol. 78, pp. 312–319.

Dodgson, K. S. & R. G. Price, 1962. A note on the determination of the ester sulfate content of sulfated polysaccharides. *Biochem. J.*, Vol. 84, pp. 106–110.

Ehrlich, J. & S. S. Stivala, 1973. The chemistry and pharmacology of heparin. *J. pharm. Sci.*, Vol. 62, pp. 517–544.

Lagunoff, D. & G. Warren, 1962. Determination of 2-deoxy-2-sulfoaminohexose content of mucopolysaccharides. *Archs Biochem. Biophys.*, Vol. 99, pp. 396–400.

Mathews, M. B., J. Duh & P. Person, 1962. Acid mucopolysaccharides of invertebrate cartilage. *Nature, Lond.*, Vol. 193, pp. 378–380.

Mejbaum, W., 1939. Estimation of small amounts of pentose especially in derivatives of adenylic acid. *Hoppe-Seyler's Z. physiol. Chem.*, Vol. 258, pp. 117–120.

Nagasawa, K., A. Ogamo & S. Hayashi, 1972. Heparinoid activities of depolymerized polysulfates. *Archs Biochem. Biophys.*, Vol. 150, pp. 451–458.

Nakanishi, K., N. Takahashi & F. Egami, 1956. Infrared spectra of charoninsulfuric acid, chondroitinsulfuric acid and some related polysaccharides. *Bull. chem. Soc. Japan*, Vol. 29, pp. 434–438.

Rahemtulla, F. & S. Lovtrup, 1974. The comparative biochemistry of invertebrate mucopolysaccharides-II. Nematoda; Annelida. *Comp. Biochem. Physiol.*, Vol. 49B, pp. 639–646.

Roden, L., J. R. Baker, A. Cifonelli & M. B. Mathews, 1972. Isolation and characterization of connective tissue polysaccharides. In, *Methods in enzymology, Vol. 28*, edited by V. Ginsburg, Academic Press, New York, pp. 73–140.

Seno, N., F. Akiyama & K. Anno, 1972. A novel dermatan polysulfate from hagfish skin, containing trisulfated disaccharide residues. *Biochim. biophys. Acta*, Vol. 264, pp. 229–233.

Soda, T., F. Egami & T. Horigome, 1940. Chondroitinsulfuric acid. I. Preparation of chondroitinsulfuric acid by use of trypaflavine. *J. chem. Soc. Japan*, Vol. 61, pp. 43–49.

Spiro, R. G., 1966. Analysis of sugar found in glycoproteins. In, *Methods in enzymology, Vol. 8*, edited by E. F. Neufeld & V. Ginsburg, Academic Press, New York, pp. 3–60.

Suzuki, S. & J. L. Strominger, 1960. Enzymatic sulfation of mucopolysaccharides in hen oviduct. *J. biol. Chem.*, Vol. 235, pp. 257–276.

Utimaru, T., 1943. Über die Schwefelsäurester in den Glucoproteinen. I, Schwefel-, Stickstoffe-, Hexosamin-, and Uronsäure-bestimmungen. *Bull. chem. Soc. Japan*, Vol. 18, pp. 235–242.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 137–145
© Elsevier/North-Holland Biomedical Press

ONTOGENY OF AN ENDOGENOUS RHYTHM IN *EURYDICE PULCHRA*

M. H. HASTINGS and E. NAYLOR
Department of Marine Biology, University of Liverpool, Port Erin, Isle of Man, U.K.

Abstract: Field catches of *Eurydice pulchra* (Leach) from an Isle of Man beach indicated that adult animals were active in the water column solely during spring tide periods, whereas juvenile animals swam abundantly at high tide irrespective of lunar phase. Under constant conditions in the laboratory, animals kept in actographs with sand showed a circa-tidal rhythm of spontaneous emergence and swimming activity. In adults, the activity was greatest during "expected" night-time high-waters and was expressed solely after collection during spring tide periods. The rhythm was independent of sex, season or reproductive status. Juveniles showed no such diurnal modulation of the rhythm and the pattern was expressed throughout the semi-lunar cycle. These ontogenetic differences in behaviour are discussed in the light of previous work on the rhythm, and in terms of the differing habits of the mature and immature animals.

INTRODUCTION

The swimming activity of *Eurydice pulchra* (Leach) during periods of tidal immersion of its sand beach habitat is well documented. In the laboratory, emergence from sand and swimming are expressed with an endogenous circa-tidal rhythm, particularly at the times of spring tides. Water turbulence appears to be an important synchronizer of the rhythm (Jones & Naylor, 1970; Fish & Fish, 1972; Alheit & Naylor, 1976). A similar rhythm, also entrained by turbulence, has been demonstrated in the closely related *Excirolana chiltoni* (Klapow, 1972a), in which newly released young were shown to exhibit an endogenous rhythmicity akin to that of the brooding female, probably as a result of marsupial experience (Klapow, 1972b). The present paper considers developmental changes in the spontaneous emergence and swimming behaviour of *Eurydice*, particularly in view of preliminary observations that immature and mature specimens appear to behave differently in the field. It also seeks to reconcile further the results of Jones & Naylor (1970) and Fish & Fish (1972), who observed different patterns of emergence from sand. Hitherto these were explained partly as apparent modulation of the circa-tidal swimming rhythm by a circa-semilunar rhythm of spontaneous emergence from the substratum (Alheit & Naylor, 1976). Possible ontogenetic differences in behaviour have not previously been considered in this context.

Materials and Methods

The population of *E. pulchra* studied was that previously investigated by Alheit & Naylor (1976) at Derbyhaven, Isle of Man. The duration of the life cycle of the species in that vicinity is 2 yr, the population consisting of mature or maturing adults identified by the presence of paired penes and silver-coloured testes in males, and developing ovaries and oostegites in females, with an immature juvenile year-class defined as animals < 3.5 mm in body length (Hastings, unpubl.).

Emergence and swimming activity in the field and laboratory were considered at four stages of development: (1) juveniles 2–4 months old (Nov.–Jan. 1978); (2) juveniles 8–10 months old, during a period of rapid somatic growth (May–July 1979); (3) newly matured adults in which gametogenesis had commenced, 14–16 months old (Nov.–Jan. 1978); and (4) mature breeding population, 20–22 months old (May–July 1979). It should be noted that animals at stages (1) and (3) and (2) and (4) were studied simultaneously and so any differences recorded in their behaviour were directly attributable to developmental stage rather than season.

Swimming in the field was quantified by 50-m plankton hauls taken at the water's edge, 1 h after the daytime high-water, using a coarse mesh D-net of 25 cm radius. Hauls were taken daily over one or more spring–neap cycles.

Laboratory recordings of swimming activity following spontaneous emergence from sand in continuous darkness were made using infra-red beam actographs coupled with a Rustrak event recorder. Two separate actograph chambers were used to duplicate every experimental run, each with 25 animals and 3–4 cm depth of clean sand. Experiments were run at constant temperature in a room at 15 °C, animals being collected at various times through the 15-day spring–neap cycle, and their activity monitored in constant conditions for 4 to 5 days. Hourly activity values were expressed as a percentage of the highest recorded value for each particular run.

The high sensitivity of the equipment necessary to register the movements of the small isopods made the recording susceptible to interference by small voltage and temperature fluctuations. Some traces were therefore interrupted but complete traces are given here which are typical of patterns of activity seen in several other uninterrupted and interrupted recordings.

Results

Field observations

The newly matured adults in winter (Fig. 1a) showed peak catches around new and full moon, with minimal numbers taken during the neap-tide period of the first quarter. This pattern was repeated in the summer (Fig. 1b) with peak catches of both males and females being phased close to the times of full and new moon, that is

around spring tides. The absence of a peak around new moon in late June was probably related to the completion of mating, after which it was observed that the ovigerous females became strongly photonegative and did not emerge during the day, whilst the males rapidly died off. The striking feature of results in Fig. 1a and b is that large catches of adults were never made during neap tide periods of the first and third quarters of the moon. These observations on the swimming rhythm of *E. pulchra* confirm and extend those of Fish & Fish (1972) and Alheit & Naylor

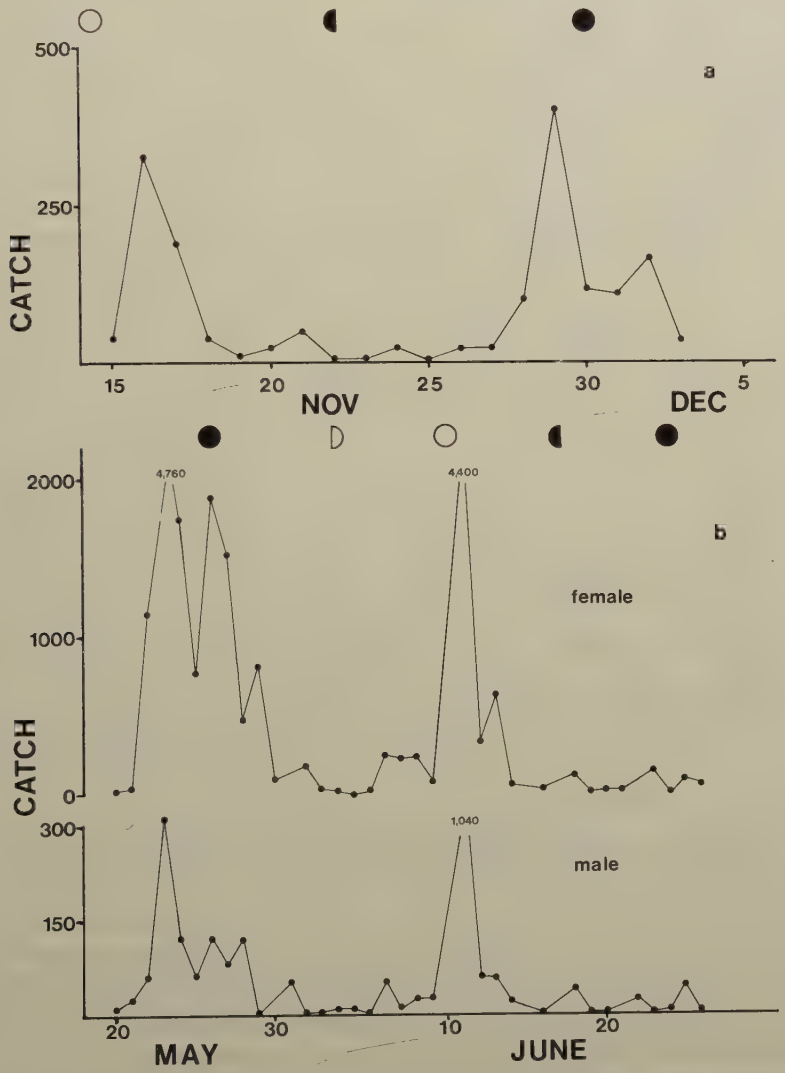


Fig. 1. Variation in catch of adult *Eurydice* in daily plankton hauls at the water's edge, 1 h after high water: a, young adults, winter 1978; b, mature adults, summer 1979.

(1976), all of which lead to the conclusion that adult males and females exhibit a semi-lunar rhythmicity of swimming in the field.

In contrast, the catches of juveniles in winter and summer (Fig. 2) were erratic. A peak after the full-moon in mid-November was not followed by another

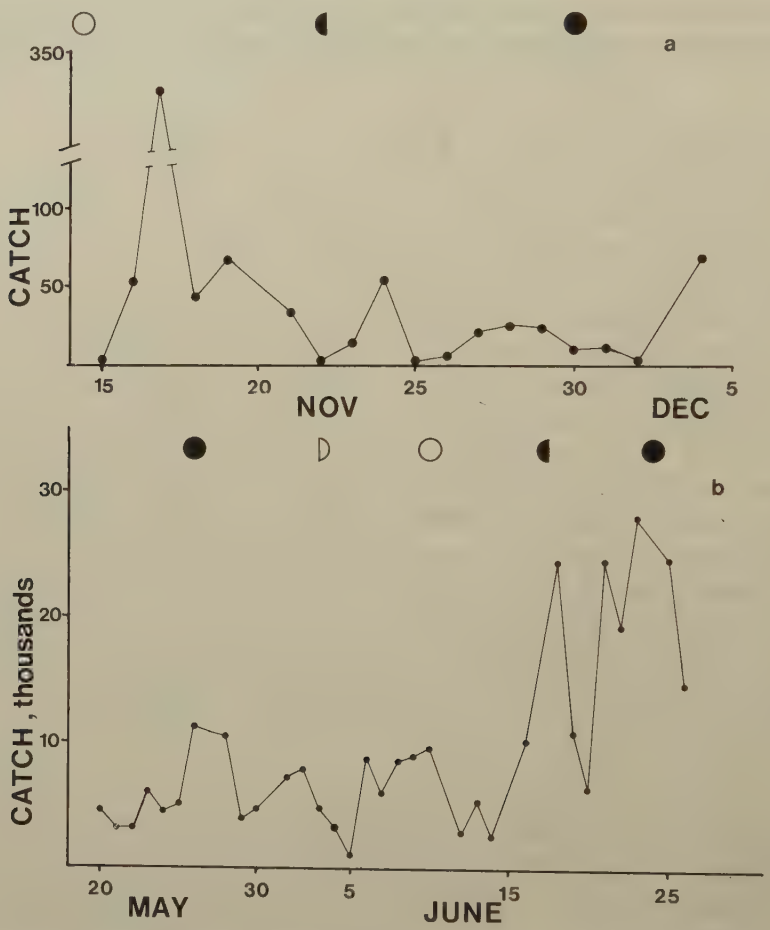


Fig. 2. Variation in catch of juvenile *Eurydice* in daily plankton hauls at the water's edge, 1 h after high water: a, newly released, winter 1978; b, 8–10 months old, summer 1979.

peak 15 days later (Fig. 2a) as it was for adults (Fig. 1a). In May and June (Fig. 2b) there were no peaks of catch of juveniles during spring tide periods equivalent to those shown by adult animals (Fig. 2b), and the low catches of adults in mid to late June coincided with increased overall catches of juveniles, presumably as they migrated to the upper shore as temperatures rose.

CONTENTS VOLUME 46

ROBERT J. FELLER, Development of the sand-dwelling meiobenthic harpacticoid copepod *Huntemannia jadensis* Poppe in the laboratory 1

PAUL J. HIGGINS, Effects of food availability on the valve movements and feeding behavior of juvenile *Crassostrea virginica* (Gmelin). II. Feeding rates and behavior 17

JOEL C. GOLDMAN and ROGER MANN, Temperature-influenced variations in speciation and chemical composition of marine phytoplankton in outdoor mass cultures 29

JEREMY D. PAUL, Upper temperature tolerance and the effects of temperature on byssus attachment in the queen scallop, *Chlamys opercularis* (L.) 41

LARS HAGERMAN, The regulation of calcium and magnesium in the urine during the moulting cycle of *Crangon vulgaris* (Fabr.) 51

KEVIN M. SHERMAN and BRUCE C. COULL, The response of meiofauna to sediment disturbance 59

W. HERBERT WILSON, JR., A laboratory investigation of the effect of a terebellid polychaete on the survivorship of nereid polychaete larvae 73

STEPHEN H. HOLANOV and JOHN R. HENDRICKSON, The relationship of sand moisture to burrowing depth of the sand-beach isopod *Tylos punctatus* Holmes and Gay 81

R. T. KNEIB, A. E. STIVEN and E. B. HAINES, Stable carbon isotope ratios in *Fundulus heteroclitus* (L.) muscle tissue and gut contents from a North Carolina *Spartina* marsh 89

CAROL SLOCUM, Differential susceptibility to grazers in two phases of an intertidal alga: advantages of heteromorphic generations 99

J. J. P. CLOKIE and A. D. BONEY, *Conchocelis* distribution in the Firth of Clyde: estimates of the lower limits of the photic zone 111

B. PATEL, J. EHRLICH, S. S. STIVALA and N. K. SINGH, Comparative studies of mucopolysaccharides from marine animals. I. *Raja eglanteria* Bosc. 127

M. H. HASTINGS and E. NAYLOR, Ontogeny of an endogenous rhythm in *Eurydice pulchra* 137

GUIDO CHELAZZI and MARCO VANNINI, Zonal orientation based on local visual cues in *Nerita plicata* L. (Mollusca: Gastropoda) at Aldabra Atoll 147

WIM ADMIRAAL and HARRY PELETIER, Distribution of diatom species on an estuarine mud flat and experimental analysis of the selective effect of stress 157

RAYMOND C. HIGHSMITH, Geographic patterns of coral bioerosion: a productivity hypothesis . . 177

J. BLACKSTOCK, Estimation of activities of some enzymes associated with energy-yielding metabolism in the polychaete, *Glycera alba* (Müller), and application of the methods to the study of the effect of organic pollution 197

JOHN C. MCNAMARA, Ultrastructure of the chromatophores of *Palaemon affinis* Heilprin (Crustacea: Decapoda). The structural basis of pigment migration 219

MARK A. RAGAN and J. S. CRAIGIE, Quantitative studies on brown algal phenols. IV. Ultraviolet spectrophotometry of extracted polyphenols and implications for measuring dissolved organic matter in sea water 231

T. D. SABOURIN and W. B. STICKLE, Respiratory and osmoregulatory responses of the hermit crab, *Clibanarius vittatus* (Bosc), to salinity changes 241

R. M. G. WELLS, P. J. JARVIS and S. E. SHUMWAY, Oxygen uptake, the circulatory system, and haemoglobin function in the intertidal polychaete *Terebella haplochaeta* (Ehlers) 255

MELBOURNE R. CARRIKER, ROBERT E. PALMER, LOWELL V. SICK and CONSTANCE C. JOHNSON, Interaction of mineral elements in sea water and shell of oysters (*Crassostrea virginica* (Gmelin)) cultured in controlled and natural systems 279

AUTHOR INDEX 297

LABORATORY RECORDINGS

In all, about 25 recordings of adult activity patterns were made. The data given are uninterrupted traces typical of the patterns seen. Fig. 3 illustrates the winter pattern of spontaneous emergence and swimming activity of newly matured *Eurydice* collected during various days of the spring–neap cycle and recorded for over 100 h in constant darkness in the laboratory. The animals clearly showed a persistent rhythm of emergence and swimming when collected during spring tides (Day 1 and 15), but spontaneous activity was much reduced and the persistence of rhythmicity very short-lived between the spring tide periods, particularly just after neap tides (Days 8 and 12).

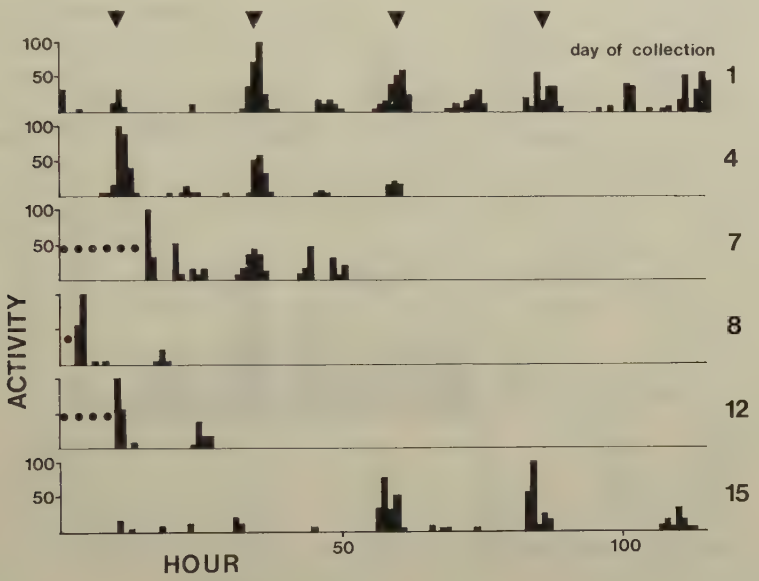


Fig. 3. Hourly activity values of young adult *Eurydice* collected on different days of semi-lunar cycle and kept under constant conditions and darkness in the laboratory actograph: traces start to the right of the closed circles; ▼ time of expected night-time high-water.

Throughout the experiments, the expressed endogenous rhythm was basically circa-tidal, with peak activity occurring mainly at the times of early ebb tide in the field, but activity was particularly high during those periods of expected high tide which occurred during the hours of expected darkness, indicating a diurnal modulation of the circa-tidal rhythm. This winter pattern was repeated in summer by the breeding adults (Fig. 4) collected at spring tides. Adult males, non-ovigerous females and ovigerous females all gave the same type of trace, indicating that the endogenous rhythm is independent of sex, season or reproductive condition and is very consistent in its form of expression. The data again display circadian modulation of the ba-

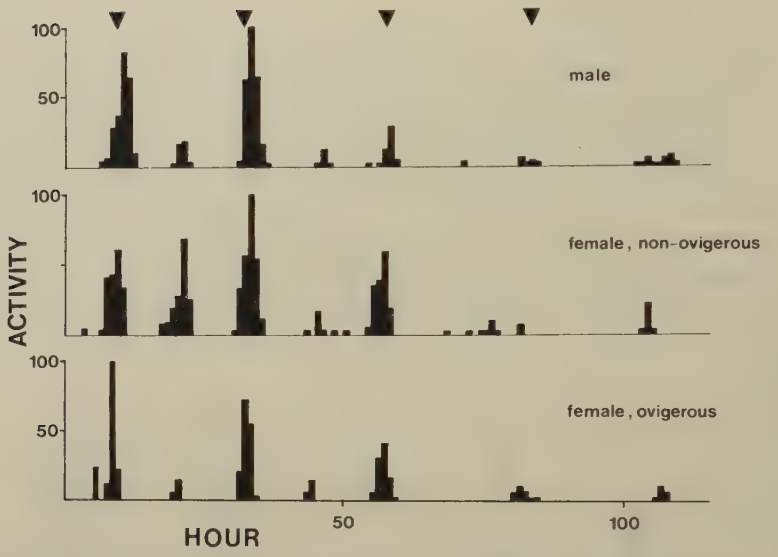


Fig. 4. Hourly activity values of mature male and female *Eurydice* collected during spring tides and kept in laboratory actographs under constant conditions and darkness: symbols as in Fig. 3.

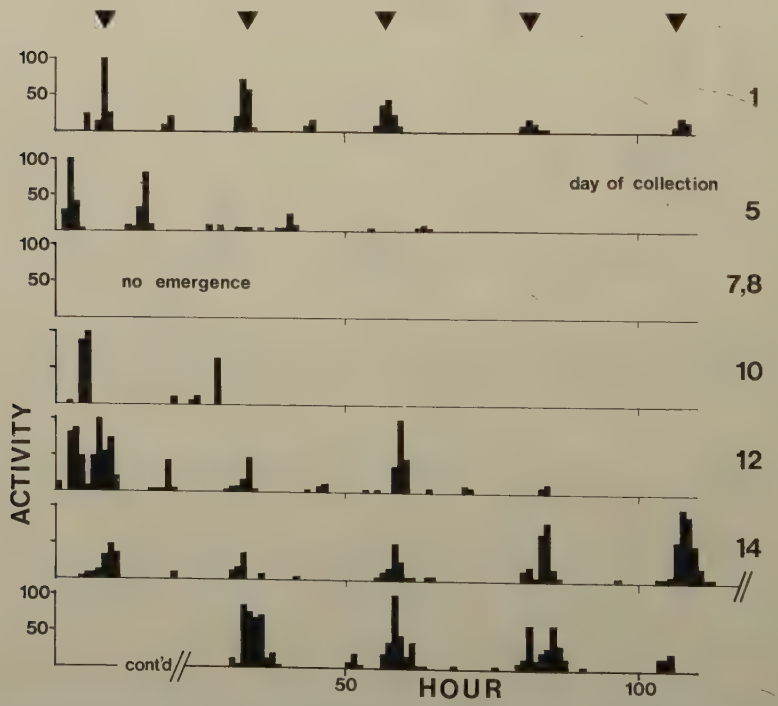


Fig. 5. Hourly activity values of ovigerous female *Eurydice* collected on different days of the semi-lunar cycle and kept in laboratory actographs under constant conditions and darkness: symbols as in Fig. 3.

sically circa-tidal rhythm. The traces for ovigerous females were continued through the semi-lunar cycle (Fig. 5) and these again showed a loss of spontaneous emergence at neap tides (Days 7, 8 and 10), with re-establishment of the rhythm during the following spring tides. This pattern is identical to that shown by young adults in winter, further emphasizing the consistency and reproducibility of the overt expression of the rhythms in the laboratory.

The activity of juveniles in their first summer, recorded under constant conditions in the laboratory at the same time as adult activity, was, however, very different. Of the eight experiments, four yielded suitable uninterrupted traces. These are given in Fig. 6 and are representative of the overall pattern. A circa-tidal

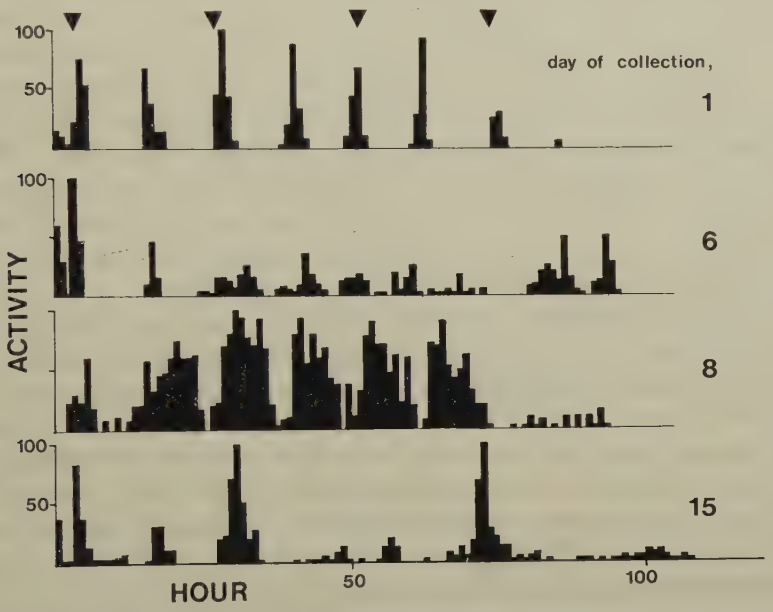


Fig. 6. Hourly activity values of juvenile *Eurydice* collected on different days of the semi-lunar cycle and kept in laboratory actographs under constant conditions and darkness: symbols as in Fig. 3.

emergence and swimming pattern, in phase with the tidal cycle on the shore is evident in these data, but there is no evidence of regular diurnal modulation of this strong rhythm. Furthermore, the level of activity and the persistence of the circa-tidal rhythm were as great during neap tide periods (Days 6 and 8) as at spring (Days 1 and 15). In juveniles, there was clearly no diurnal or semilunar modulation of the circa-tidal pattern such as occurred in adults, and the circa-tidal rhythm was consistently expressed for several days in each individual recording.

Discussion

The results of the present experiments accurately reflect the field observations, with adult animals being active mainly at spring tides, a pattern of activity well documented for *E. pulchra* by Fish & Fish (1972), Alheit & Naylor (1976), as well as for *Excirolana* (Klapow, 1972) and *Pseudaega* (Fincham, 1973). In contrast, juveniles swim irrespective of lunar phase or time of day showing a persistent pattern of spontaneous emergence from the sediment at the time of expected high tide. Overall, the juveniles show a greater level of sustained spontaneous swimming activity which in the field affords greater opportunity for feeding. It is known that small *Eurydice* show a relatively higher metabolic rate than large specimens (Jones, 1970). Similarly, Johnson (1976) calculated energy budgets for juvenile and adult *Cirolana harfordi* and concluded that respiration accounted for 78% of assimilated energy in juveniles, but only 59% in adults. The gut morphology of *Eurydice* is adapted to accommodate large meals, which are followed by periods of digestion with the animal at rest (Jones, 1968), and this is a mode of feeding which is probably most efficient in large adults. Rapidly growing juveniles with a higher rate of metabolism would be expected to feed more often than adults, and frequent emergence and feeding in the water column would, therefore, appear most appropriate for the juveniles, despite the risk of predation at the times of feeding.

In contrast, somatic growth slows once adult size is attained, the relative metabolic rate is lowered and a meal large enough to sustain an individual for several days can be obtained in a short time. Adults, particularly females, have little to gain by extensive swimming excursions once mating is achieved, and investment in growth and reproduction would be threatened by predation. Restricted emergence interspersed by long periods burrowed in the sediment would clearly seem to be more appropriate. A synchronous restricted emergence pattern throughout the adult population would also facilitate copulation between individuals which follows pair formation in the water column in early summer.

The greater swimming and emergence activity during "expected" night in the laboratory correlates with field observations of nocturnal activity by *Eurydice* (Fish & Fish, 1972; Jones & Naylor, 1970; Alheit & Naylor, 1976). In the northern Irish Sea, night-time high-water occurs during spring tide periods, while neap high-waters are at dawn and dusk. A pattern of emergence at night-time high-water would, therefore, be restricted to the times of spring tides and be expressed as a circa-semilunar rhythm.

The spontaneous emergence and swimming of *Eurydice* in the laboratory contrasts with the report of Jones & Naylor (1970), where it was not observed. Alheit & Naylor (1976), using animals from the locality investigated by Jones & Naylor, did, however, show spontaneous emergence from sand during spring tide periods, and suggested that the earlier observations could be explained since those experiments were carried out during periods of neap tides, when such emergence would

not be expected. The emergence and swimming pattern of adults recorded here agrees with the findings of Fish & Fish (1972) and Alheit & Naylor (1976), although the pronounced diurnal modulation of the endogenous circa-tidal activity rhythm noted here has not previously been recorded under conditions of constant darkness. It is apparent that an endogenous circa-tidal rhythm of emergence and swimming is a feature of all *E. pulchra* populations so far studied. The dawn and dusk timing of spring high-waters at the site studied by Jones & Naylor (1970) may explain the absence of a diurnal modulation of emergence and swimming in the laboratory. The fact that the circa-tidal rhythm is, however, expressed throughout the semi-lunar cycle by juvenile *Eurydice*, but only at spring tides by adults, is quite new. In earlier papers, the adaptive significance of the emergence and swimming rhythm of *E. pulchra* has been discussed in the context of the maintenance of zonation patterns on the shore (Jones & Naylor, 1970; Fish & Fish, 1972; Alheit & Naylor, 1976). In the present populations (Hastings, unpubl.), as elsewhere, the upper limit of zonation rises and falls throughout the spring–neap cycle, and the question has been posed as to how movement downshore occurs on falling springs to avoid stranding high on the shore at neap tides (Alheit & Naylor, 1976). As proposed by those authors, the semi-lunar swimming rhythm, with peak activity at times of reducing spring high tides, is clearly a mechanism permitting the avoidance of neap-stranding of adults. The absence of semi-lunar rhythmicity of swimming in juveniles is apparently inconsistent with that hypothesis, but the results presented here suggest that the juveniles, with a feeding habit involving repeated emergence during successive high tides, would in any case continuously adjust the upper limit of their distribution on the shore.

References

Alheit, J. & Naylor, E., 1976. Behavioural basis of intertidal zonation in *Eurydice pulchra* Leach. *J. exp. mar. Biol. Ecol.*, Vol. 23, pp. 135–144.

Fincham, A. A., 1973. Rhythmic swimming behaviour of the New Zealand sand beach isopod *Pseudaega punctata* Thomson. *J. exp. mar. Biol. Ecol.*, Vol. 11, pp. 229–237.

Fish, J. D. & S. Fish, 1972. The swimming rhythm of *Eurydice pulchra* and a possible explanation of intertidal migration. *J. exp. mar. Biol. Ecol.*, Vol. 8, pp. 195–200.

Johnson, W. S., 1976. Population energetics of the intertidal isopod *Cirolana harfordi*. *Mar. Biol.*, Vol. 36, pp. 351–357.

Jones, D. A., 1968. The functional morphology of the digestive system in the carnivorous intertidal isopod *Eurydice*. *J. Zool., Lond.*, Vol. 156, pp. 363–376.

Jones, D. A., 1970. Factors affecting the distribution of the intertidal isopods *Eurydice pulchra* Leach and *E. affinis* Hansen in Britain. *J. Anim. Ecol.*, Vol. 39, pp. 455–472.

Jones, D. A. & E. Naylor, 1970. The swimming rhythm of the sand beach isopod *Eurydice pulchra*. *J. exp. mar. Biol. Ecol.*, Vol. 4, pp. 188–199.

Klapow, L. A., 1972a. Natural and artificial rephasing of a tidal rhythm. *J. comp. Physiol.*, Vol. 79, pp. 233–258.

Klapow, L. A., 1972b. Fortnightly molting and reproductive cycles in the sand beach isopod *Excirolana chiltoni*. *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 143, pp. 568–591.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 147–156
© Elsevier/North-Holland Biomedical Press

ZONAL ORIENTATION BASED ON LOCAL VISUAL CUES IN *NERITA PLICATA* L. (MOLLUSCA: GASTROPODA) AT ALDABRA ATOLL[1]

GUIDO CHELAZZI and MARCO VANNINI
Istituto di Zoologia dell'Università, Firenze, Italy

Abstract: *Nerita plicata* L. from Aldabra Atoll orientated coastwards when released in front of all coasts irrespective of their compass bearing. Correct coastward orientation was achieved within a small circular arena placed in front of the coast but orientation was at random when the arena was placed on top of a coastal dune where all landscape cues were eliminated. Tests with the sun in opposite azimuth (early morning and late afternoon) revealed a negative phototaxis which barely altered orientation towards the local coast. None of the tests indicated the presence of a chronometric correction of solar azimuth variation. *N. plicata* thus employs local information, activating either a contour-related orientation or one based on the evaluation of differences in low lying illumination between sea and land hemicycles.

INTRODUCTION

What little is known about the mechanisms employed by molluscs in zonal orientation concerns mainly neritid and littorinid gastropods. Coastward orientation was shown in dislodged *Nerita plicata* by Warburton (1973) and in *N. textilis* by Chelazzi & Vannini (1976). Warburton concludes that solar orientation, using a time controlled reversing phototaxis, is involved in the zonal orientation of *N. plicata* which, under the local conditions (rapid variation of the solar azimuth around mid-day) allows the animals to maintain a constant coastward orientation, while Chelazzi and Vannini maintain that *N. textilis* performs a visual orientation involving local cues.

Warburton's (1973) conclusion that *N. plicata* employs solar orientation has been widely quoted (Jander, 1975; Newell, 1976; Gendron, 1977; Hoffman *et al.*, 1978; Underwood, 1979) but, in our opinion, his data demonstrate only a capacity to orientate coastwards and do not constitute proof that this capacity is based on a time-controlled reversing phototaxis with the sun as the primary directional cue. The present paper attempts to verify experimentally the existence of a true solar orientation in *N. plicata* L. through a series of tests in front of both the native and differently orientated shores.

[1] Publication of the Centro di Studio per la Faunistica ed Ecologia Tropicali del Consiglio Nazionale delle Ricerche.

Materials and Methods

Tests were conducted on Aldabra Atoll, Indian Ocean, in February–March 1979. Detailed information on the coastal morphology, zonation and notes on the ecology of *N. plicata* in the collecting and test sites have been given by Hughes (1971) and Taylor (1971).

DISPLACEMENT EXPERIMENTS

Displacement experiments were done in three differently orientated shores by recording the direction of movement of animals collected on the rocky coast at about mean high water springs and released 30–50 m from the cliffs on the low eulittoral platforms. The animals were collected and tested at Ile Picard (in front of the Settlement) and on the northeast (Anse Cèdres) and southern (Dune Patates) coast of Grande Terre. One hundred animals from each locality were collected, marked with a quick drying, water resistant paint and immediately released. Collection and release took place during the same diurnal low water; the position of the snails with respect to the point of release was recorded 24 h later.

ARENA EXPERIMENTS

Arena experiments were done at Anse Cèdres, Dune Patates and on Ile Picard. Animals were released one at a time in the centre of a semi-transparent polystyrene disc 0.08 mm thick and 40 cm in diameter set at a depth of 3 mm within a Plexiglas plane 46 cm in diameter, which was unscreened and compass orientated according to the references on its rim. A fresh polystyrene disc was used for each animal in order to eliminate any trail-following effect by successive specimens. Snails were released orientated at random in one of the four principal directions of the plane. The animals were kept immersed in sea water until tested in order to keep them active. All the diurnal tests were conducted under the visible sun while the nocturnal tests were made at new moon.

The non-uniformity of the circular distributions and the significance of clustering around the expected direction were tested respectively with the Rayleigh and the V test (see Batschelet, 1965, 1972). Two-sample difference was analysed using the Watson's U^2 test (see Batschelet, 1972), while a comparison between three distributions was done with the Watson & Williams multisample test (see Schmidt-Koenig, 1975).

Results

DISPLACEMENT EXPERIMENTS

The test localities and directions taken by the animals during 24 h are shown in Fig. 1. Each distribution differs statistically from a uniform one (Rayleigh test:

$P_z < 0.001$) and there is significant clustering around the expected direction (V test: $P_u < 0.0001$). The mean directions of the three samples, tested with the Watson & Williams multisample test, differ significantly from each other ($P < 0.001$).

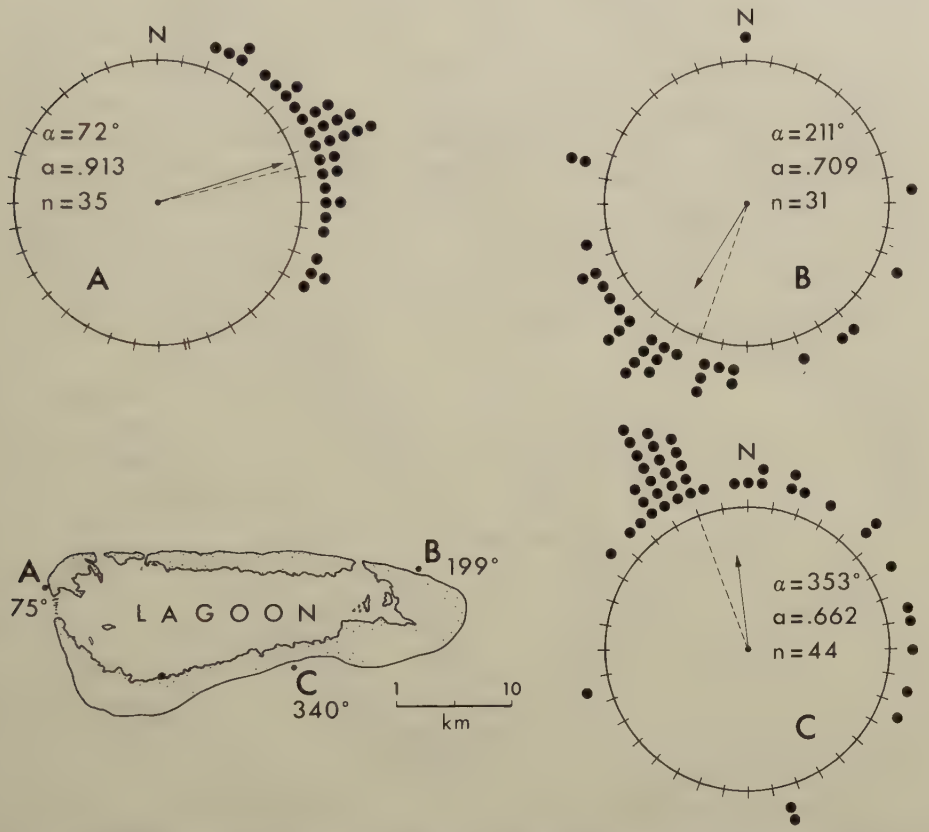


Fig. 1. Displacement experiments with *Nerita plicata*: map shows the test sites (A C) on Aldabra Atoll and expected (shoreward) direction at each locality; ●, recovery directions of the animals; α, average direction; a, vector length; n, number of recovered animals; dashed line, shoreward direction.

Despite the bright paint used in marking the specimens, only part of each group was sighted 24 h later (35% at the Settlement, 31% at Anse Cèdres, and 44% at Dune Patates). The unsighted animals must have been either hidden between cracks in the rock and algae or dislodged by waves during high water. In 24 h the sighted snails travelled an average of 174 cm at Settlement, 271 cm at Anse Cèdres, and 80 cm at Dune Patates. There is no apparent correlation between chosen direction and distance covered. Continued observations of displaced "native" snails at the Settlement confirmed that the majority of visible snails reached their zone of origin within 5 days.

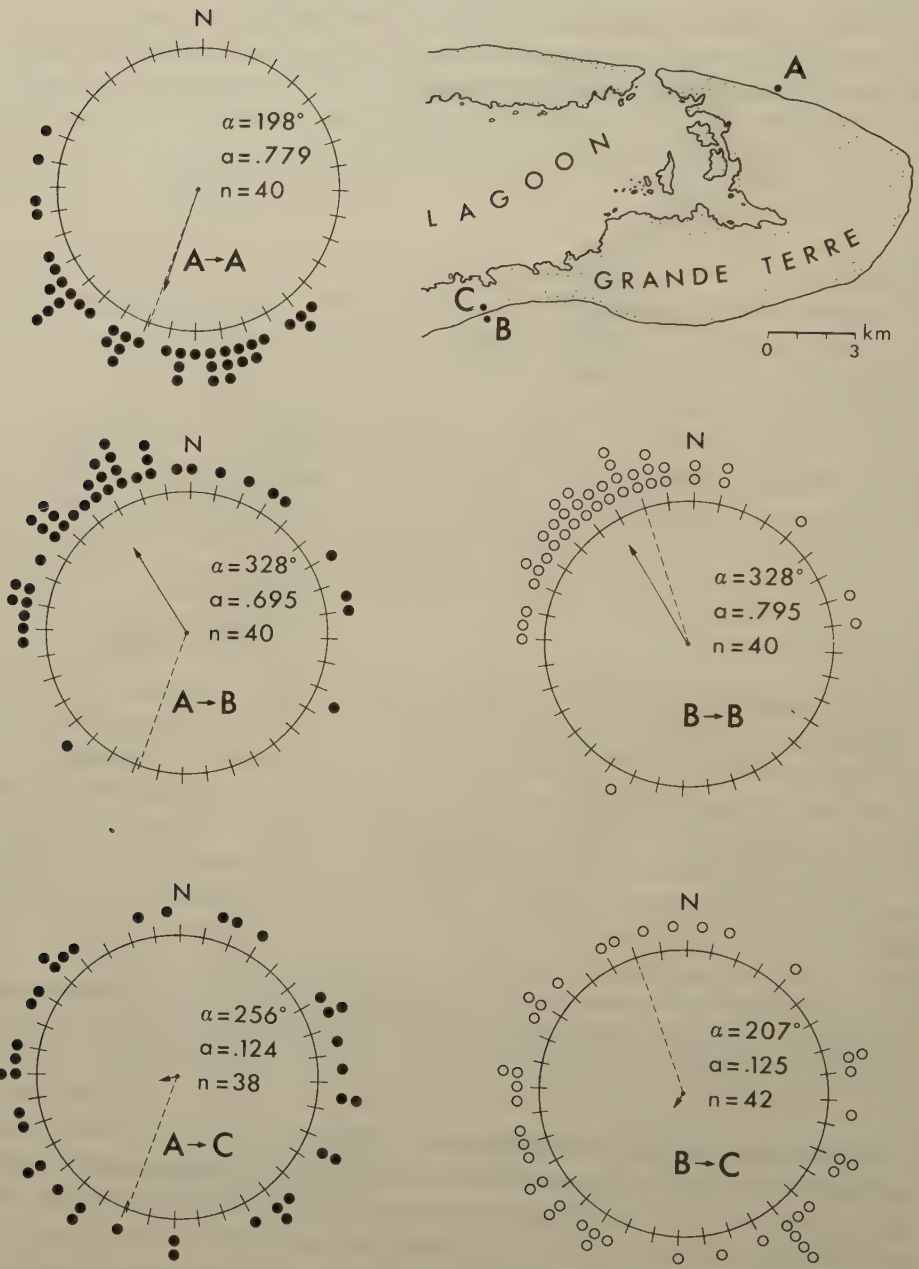


Fig. 2. Daytime arena experiments with *Nerita plicata*: map shows the eastern part of Aldabra Atoll and test site in front of Anse Cèdres (A) and Dune Patates (B), and on top of the dune at Dune Patates (C); ●, animals collected at Anse Cèdres; ○, animals collected at Dune Patates; dashed line, shoreward direction at the collecting site; the two capital letters within each diagram indicate respectively the collecting and test site; for further explanation see Fig. 1.

ARENA EXPERIMENTS

The distribution of directions moved by animals collected at Anse Cèdres (Fig. 2, A → A) and Dune Patates (Fig. 2, B → B) tested in front of their home coast is significantly non-uniform ($P_z < 0.001$) and clustered around the shoreward direction at both localities ($P_u < 0.0001$).

In order to test whether the preferred direction would remain constant despite the direction of the coast at the test site or would vary according to the direction of test shore, a group of animals from Anse Cèdres was tested at Dune Patates. The animals, placed in plastic containers with rocks and water on the bottom, were carried over the atoll lagoon to the test site. Tests were made 24 and 48 h after collection. The distribution (Fig. 2, A → B) is statistically different from a uniform one ($P_z < 0.001$) and significantly clustered around the new local shoreward direction ($P_u < 0.0001$) but not around that of the home coast ($P_u > 0.10$). Thus the

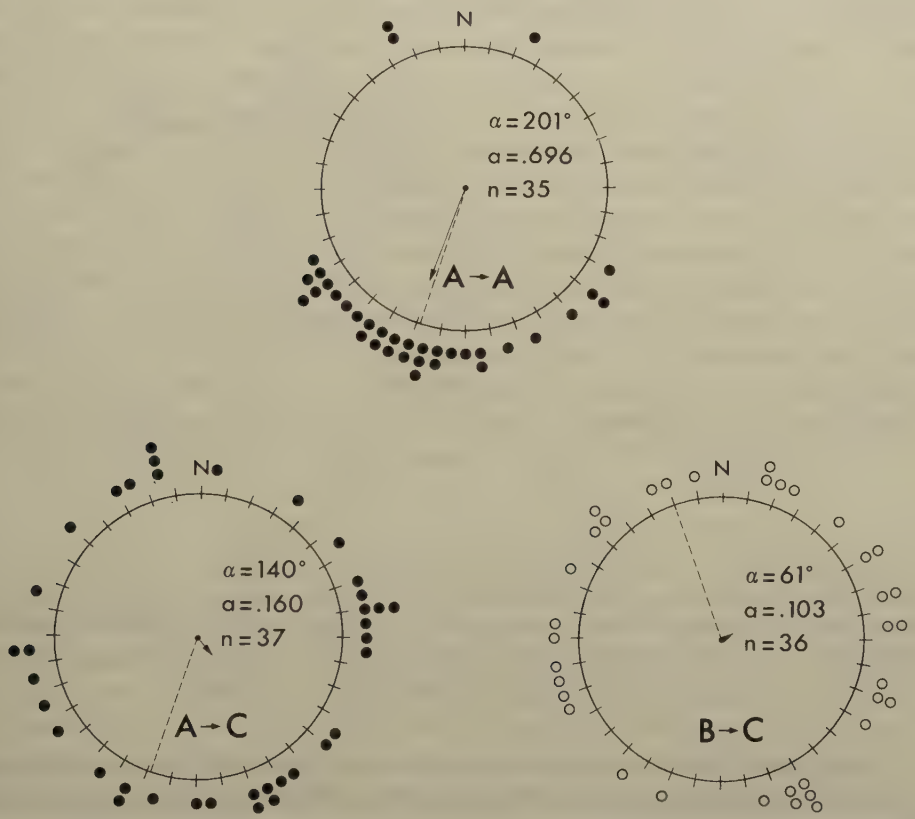


Fig. 3. Night-time experiments with *Nerita plicata*: ●, animals collected at Anse Cèdres; ○, animals collected at Dune Patates; the capital letters within each diagram refer to sites shown in the map of Fig. 2; for further explanation see Fig. 1.

orientation of the transported animals did not differ from that of the native (Fig. 2, B → B) animals ($U^2_{40,40} = 0.04$; $P > 0.10$), but was shifted by 130° with respect to that of snails on the shore of origin.

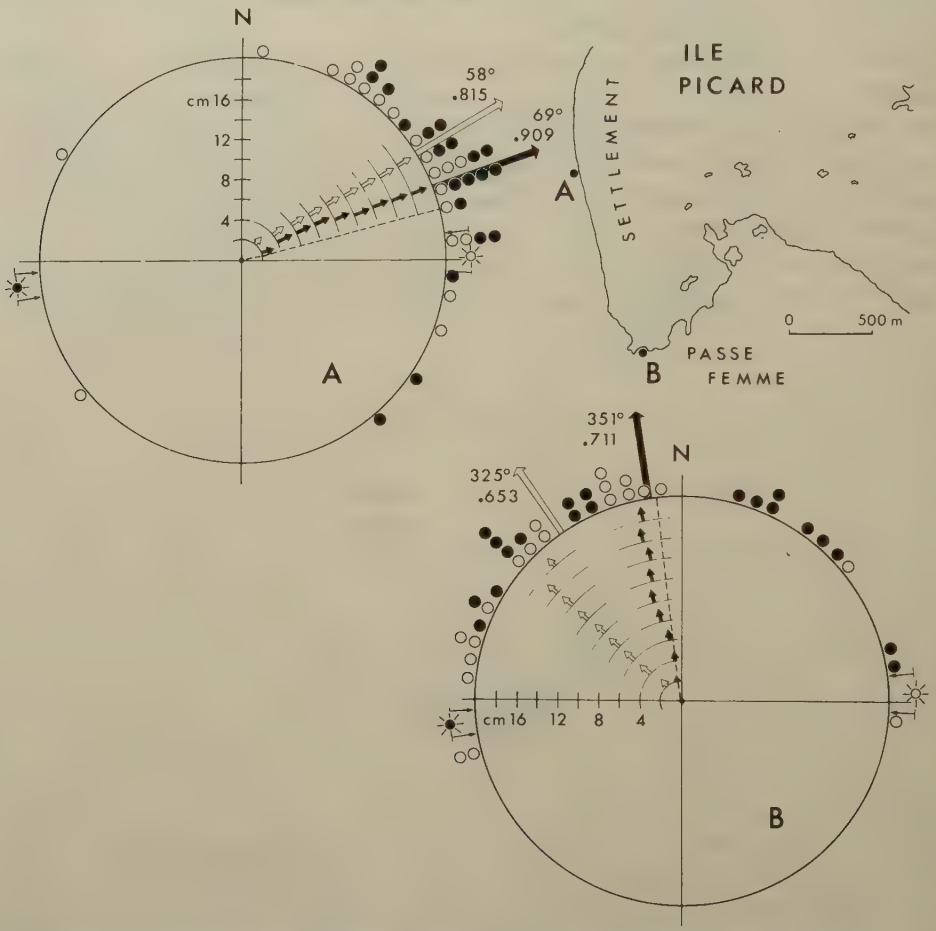


Fig. 4. Morning–afternoon arena experiments with *Nerita plicata*: map shows the collection-test site at Settlement (A) and test site at Passe Femme (B); open symbols, morning tests; black symbols, afternoon tests; dots, headings of animals reaching the arena border; outer arrows, resultant vectors of the morning and afternoon distributions; inner arrows, resultant vectors at 2–18 cm from the arena centre; also shown is the sun's azimuth during testing.

A combined group of snails from Anse Cèdres and Dune Patates was then tested on top of a coastal dune about 100 m inland from the shore line at Dune Patates. The point chosen was higher than the surroundings so that all landscape cues were invisible to the animals in the arena, while they had full access to celestial cues. Under these conditions distribution of movement directions (Fig. 2, A → C and

B → C) was not significantly different from a uniform one ($P_z > 0.10$) and there was no clustering in the expected direction ($P_u > 0.10$). There was no statistically significant difference between the distribution of the two groups within the arena ($U^2_{38,42} = 0.03$; $P > 0.10$).

Animals collected and tested at Anse Cèdres during nocturnal low water in front of the shore (Fig. 3, A → A) orientated shorewards ($P < 0.001$; $P_u < 0.0001$) with the same precision as did those tested during the day ($U^2_{40,35} = 0.10$; $P > 0.10$). The distribution within the arena of snails collected at Anse Cèdres and Dune Patates tested on top of the dune (Fig. 3, A → C and B → C), did not differ from a uniform one ($P_z > 0.10$) and neither was there any statistical difference between the distributions of the two groups within the arena ($U^2_{37,36} = 0.53$; $P > 0.10$).

Further diurnal tests were made using animals freshly collected from the beachrock in front of the Settlement. Trials were conducted at the collecting site (coastward direction 75°), where the shore line is slightly convex backed by high vegetation (coconut trees and casuarines) and at Passe Femme (325°), where it is markedly concave backed by a rocky cliff topped with a low bush. Tests took place during morning (0730–0930 h) and afternoon (1630–1800 h) low water, thus with the sun in practically opposite azimuth.

There was a significant difference between the overall distribution of movement direction ($U^2_{40,40} = 1.03$; $P < 0.005$) observed in the two localities (Fig. 4). In both cases the general distributions (morning plus afternoon) of the movement directions at 20 cm from the centre of the arena differed statistically from a uniform one ($P_z < 0.001$) and were significantly clustered in the sector corresponding to the local shoreward direction ($P_u < 0.0001$). In each locality the morning distribution of directions was shifted counterclockwise with respect to the afternoon distribution. At both the Settlement and Passe Femme orientation in the afternoon tests was closer to the expected direction and there was less dispersion of the individual movement directions but these temporal trends were not statistically significant (Settlement $U^2_{20,20} = 0.08$; $P > 0.10$; Passe Femme $U^2_{20,20} = 0.10$; $P > 0.10$).

Discussion

Photomenotaxis, or light compass reaction without chronometric correction of the orientation angle, was first demonstrated in the opisthobranch *Elysia viridis* (Fraenkel, 1927; Fraenkel & Gunn, 1961). Subsequently, a similar light compass reaction has been claimed for several littoral molluscs (Newell, G. E., 1958a, b; Brafield & Newell, 1961; Newell, R., 1962; Crisp, 1969). Such a response assures the maintenance of rectilinear paths for brief periods under natural conditions but over longer periods, even if aided by a rhythmic inversion of the orientation angle, photomenotaxis can only guarantee U-shaped excursions and these are not necessarily normal to the shore line. A true zonal orientation through shifts perpendicular to

the shore (periodic migrations or recovery of the normal zone of occurrence after displacement) requires a chronometric correction of the changing azimuth of the natural light sources (sun or moon).

Littoral organisms capable of chronometric astromenotaxis (Pardi, 1979) are potentially able to maintain a direction perpendicular to the home shore at any time of day, even if placed in front of differently orientated shores or denied local visual cues, as demonstrated in littoral amphipods by Pardi & Papi (1953). The information regulating the width of the orientation angle with respect to the solar azimuth which enables the animals to assume the adaptive direction (which may be different from beach to beach) is either acquired phylogenetically as in *Talitrus saltator* and other littoral amphipods (Pardi *et al.*, 1957) or memorized from preceding experience as in *Arctosa perita* and other riparian spiders (Papi *et al.*, 1957).

Our tests exclude the possibility of such a mechanism operating in *Nerita plicata* as there was no evidence of a directional constancy with regard to shore of origin. This gastropod orientates coastward at the test site even on shores orientated differently from the original site and shows a random orientation in the absence of visible landscape cues. This behaviour is similar to that described in *N. textilis* (Chelazzi & Vannini, 1976) and in *Littorina irrorata* (Hamilton, 1978). In our opinion *Nerita plicata* uses a visual mechanism based either on a contour-related orientation (Creutzberg, 1975) or on differences in the low-lying illumination of the terrestrial and marine hemicycle. We believe that this interpretation is also applicable to Warburton's (1973) data because he makes no mention of any screen shielding the landscape cues from the sight of the animals and neither does he include any results of experiments critical to the solar hypothesis. Warburton states that deflecting or reversing the sun's rays with a mirror can induce *N. plicata* to shift its track accordingly, unfortunately without reporting the values of angular deviations, and offers evidence that orientation efficiency increased with the brightness of the sun. The shifting effect could be related to deviations from the coastward direction produced by a phototactic response to the sun, although this remains negative through the day and is of no use in orientating coastward. Such an effect was demonstrated in our morning–afternoon experiments. Orientation which improves when the sun is brightest could be due to the greater contrast between the light reflected from the terrestrial and marine horizons although our nocturnal trials refute such a result.

In conclusion, a true solar orientation using chronometric astromenotaxis has yet to be demonstrated in molluscs (see also Hamilton, 1978). On the other hand, gravity (Evans, 1961), wave movement (Gendron, 1977), and local visual cues (Evans, 1961; Chelazzi & Vannini, 1976; Hamilton, 1978) are resources used by various littorinids and neritids in zonal orientation. Future research should ascertain whether *Nerita* orientates by a true form of vision or by evaluating quantitative and/or qualitative (brightness, chromatic spectrum, polarized light) differences between light reflected off the marine and terrestrial hemicycles.

Acknowledgements

We wish to thank the Royal Society for having allowed us the opportunity to conduct research on the Atoll. Our gratitude is also extended to the staff of the Royal Society Aldabra Research Station for their help in conducting research, and in particular to Mr. C. Peet for his aid in registering data. We are indebted to Professors L. Pardi and A. Ercolini for their criticism and advice in the preparation of this manuscript.

References

Batschelet, E., 1965. *Statistical methods for the analysis of problems in animal orientation and certain biological rhythms*. Washington, D.C., The American Institute of Biological Sciences, 57 pp.

Batschelet, E., 1972. Recent statistical methods for orientation data. In, *Animal orientation and navigation*, edited by S. R. Galler *et al.*, Washington, D.C., pp. 61–91.

Brafield, A. E. & G. E. Newell, 1961. The behaviour of *Macoma balthica* (L.). *J. mar. biol. Ass. U.K.*, Vol. 41, pp. 81–87.

Chelazzi, G. & M. Vannini, 1976. Research on the coast of Somalia. The shore and the dune of Sar Uanle. 9. Coastward orientation after displacement in *Nerita textilis* Dillwyn (Gastropoda Prosobranchia). *Monitore zool. ital. (N.S.) Suppl.*, Vol. 8, pp. 161–178.

Creutzberg, F., 1975. Orientation in space: animals. Invertebrates. *Mar. Ecol.*, Vol. 2, pp. 555–655.

Crisp, M., 1969. Studies on the behavior of *Nassarius obsoletus* (Say) (Mollusca; Gastropoda). *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 136, pp. 355–373.

Evans, F., 1961. Responses to disturbance of the periwinkle *Littorina punctata* (Gmelin) on a shore in Ghana. *Proc. zool. Soc. Lond.*, Vol. 137, pp. 393–402.

Fraenkel, G., 1927. Die Photomenotaxis von *Elysia viridis*. *Z. vergl. Physiol.*, Vol. 6, pp. 385–401.

Fraenkel, G. S. & D. L. Gunn, 1961. *The orientation of animals*. Dover Publ., New York, 376 pp.

Gendron, R. P., 1977. Habitat selection and migratory behaviour of the intertidal gastropod *Littorina littorea* (L.). *J. Anim. Ecol.*, Vol. 46, pp. 79–92.

Hamilton, P. V., 1978. Adaptive visually-mediated movements of *Littorina irrorata* (Mollusca: Gastropoda) when displaced from their natural habitat. *Mar. Behav. Physiol.*, Vol. 5, pp. 255–272.

Hoffman, D. L., W. C. Homan, J. Swanson & P. J. Weldon, 1978. Flight responses of three congeneric species of intertidal gastropods (Prosobranchia: Neritidae) to sympatric predatory gastropods from Barbados. *Veliger*, Vol. 21, pp. 293–296.

Hughes, R. N., 1971. Notes on the *Nerita* (Archaeogastropoda) populations of Aldabra Atoll, Indian Ocean. *Mar. Biol.*, Vol. 9, pp. 290–299.

Jander, R., 1975. Ecological aspects of spatial orientation. *Ann. Rev. Ecol. Syst.*, Vol. 6, pp. 171–188.

Newell, G. E., 1958a. The behaviour of *Littorina littorea* (L.) under natural conditions and its relation to position on the shore. *J. mar. biol. Ass. U.K.*, Vol. 37, pp. 229–239.

Newell, G. E., 1958b. An experimental analysis of the behaviour of *Littorina littorea* (L.) under natural conditions and in the laboratory. *J. mar. biol. Ass. U.K.*, Vol. 37, pp. 241–266.

Newell, R., 1962. Behavioural aspects of the ecology of *Peringia* (= *Hydrobia*) *ulvae* (Pennant) (Gastropoda, Prosobranchia). *Proc. zool. Soc. Lond.*, Vol. 138, pp. 49–75.

Newell, R. C., 1976. Adaptations to intertidal life. In, *Adaptation to environment: essays on the physiology of marine animals*, edited by R. C. Newell, Butterworths, London, pp. 1–82.

Papi, F., L. Serretti & S. Parrini, 1957. Nuovo ricerche sull'orientamento ed il senso del tempo di *Arctosa perita* (Latr.) (Araneae-Lycosidae). *Z. vergl. Physiol.*, Vol. 39, pp. 531–561.

Pardi, L., 1979. Orientamento degli animali. In, *Enciclopedia del 900, Vol. 4*, Istituto dell'Enciclopedia Italiana, Roma, pp. 965–988.

Pardi, L., A. Ercolini, V. Marchionni & C. Nicola, 1957. Ricerche sull'orientamento degli Anfipodi del litorale: il comportamento degli individui allevati in laboratorio sino all'abbandono del marsupio. *Atti Accad. Sci., Torino*, Vol. 92, pp. 1–8.

PARDI, L. & F. PAPI, 1953. Ricerche sull'orientamento di *Talitrus saltator* (Montagu) (Crustacea–Amphipoda). I. L'orientamento durante il giorno in una popolazione del litorale tirrenico. *Z. vergl. Physiol.*, Vol. 35, pp. 459–489.

SCHMIDT-KOENIG, K., 1975. *Migration and homing in animals*. Springer Verlag, Berlin, 99 pp.

TAYLOR, J. D., 1971. Intertidal zonation at Aldabra Atoll. *Phil. Trans. R. Soc. Ser. B*, Vol. 260, pp. 173–213.

UNDERWOOD, A. J., 1979. The ecology of intertidal gastropods. *Adv. mar. Biol.*, Vol. 16, pp. 111–210.

WARBURTON, K., 1973. Solar orientation in the snail *Nerita plicata* (Prosobranchia: Neritacea) on a beach near Watamu, Kenya. *Mar. Biol.*, Vol. 23, pp. 93–100.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 157–175
© Elsevier/North-Holland Biomedical Press

DISTRIBUTION OF DIATOM SPECIES ON AN ESTUARINE MUD FLAT AND EXPERIMENTAL ANALYSIS OF THE SELECTIVE EFFECT OF STRESS[1]

WIM ADMIRAAL and HARRY PELETIER

Department of Systematic Botany, State University of Groningen, 9750 AA Haren, The Netherlands

Abstract: The distribution patterns of seven dominant diatom species on an estuarine mud flat transect were related to salinity gradients and organic pollution. The temporal distribution of species can be explained partly by the seasonal variation in irradiance and temperature, and partly by the large discharges of organically polluted fresh water during the late autumn. The selective effects of stress factors, such as low or high salinity, high concentrations of ammonia and free sulphide, and high temperatures were studied by measuring: (1) the tolerance of natural diatom populations isolated from the sediment; (2) the tolerance and capacities of unialgal cultures; and (3) the effects of the stress factors on the species composition of the populations in sediment samples incubated in the laboratory. A low salinity (1‰), but not a high salinity (20‰), markedly altered the species composition of natural diatom populations kept in the laboratory. *Navicula salinarum* Grunow and *N.* cf. *cryptocephala* Kützing outcompete other species only at a salinity of 1‰, which is below the optimum salinity for growth of these species. High concentrations (2–4 mM) of ammonia inhibited the photosynthesis of *N. phyllepta* Kützing and *N. flanatica* Grunow and decreased the cell numbers of these species in mixed populations kept in the laboratory. *N. salinarum* and *Gyrosigma fasciola* (Ehr) Cleve were relatively ammonia-tolerant and reached their highest abundance during periods of large discharges of organically polluted water. The populations on the mud-flat stations that had black, sulphide-containing layers 1 mm below the surface of the sediment, were dominated by the relatively sulphide-tolerant *Navicula salinarum* and *N. pygmaea* Kützing.

High values of irradiance and temperature were a selective factor explaining the absence of *N. flanatica* in summer. Uptake of organic substrates and the inhibitory effect of high population densities are discussed in regard to their possible modifying influence on the distribution of diatom species on the mud flat.

INTRODUCTION

Distribution patterns of diatom species on intertidal mud and sand flats are the result of the complicated interactions of these algae with manifold abiotic and biotic factors. Very little is known about the physiological characteristics of these diatom species and their relation with the physical or chemical conditions of their habitat.

In the present work we have concentrated on the diatom populations inhabiting a rather exceptional intertidal mud flat, in order to study a few factors responsible for their spatial and temporal distribution. The mud flat is in a very sheltered part of the Eems–Dollard Estuary into which large amounts of organic waste water are dis-

[1] Publication No. 24 of the project "Biological Research in the Eems-Dollard Estuary".

charged (see Fig. 1). On parts of the mud flat the reduced layers reach the sediment surface as a result of bacterial activity. The input of polluted fresh water in the vicinity of the mud flat is the cause of salinity gradients and exposure to reduced conditions on the mud flat. We assumed that in this habitat the distribution of diatom species might be regulated in the first place by stress factors caused by reduced conditions and salinity fluctuations.

In this paper the gradient in species composition of the epipelic diatom populations on the mud flat and its seasonal variation will be considered. The selective influence of environmental stress factors on the diatom communities was tested, using natural communities in the laboratory and the results compared with those of previous studies on unialgal cultures of benthic diatoms.

Material and Methods

DESCRIPTION OF THE MUD FLAT

The mud flat studied is located in the inner part of the Eems–Dollard Estuary, a part of the Dutch–German Wadden Sea (Fig. 1). Because of its sheltered and inward position in the estuary there is much deposition of fine silt; the sediment is very soft and rich in organic matter. The level of the mud flat is ≈ 1 m above the mean water level and the mean tidal amplitude is ≈ 3 m; the sediment is, therefore, exposed to the air most of the time. A transect over the mud flat was investigated (Fig. 1). The stations on this transect do not show appreciable differences in level, grain size, and total organic carbon content. The three stations nearest to the coast are, however, drained more intensively by gullys and hence are occasionally prone to desiccation, whereas other stations are not. The mud flat is devoid of macrophytes. Macro-zoobenthos is sparse, while meiofauna is often very numerous (L. Bouman, pers. comm.). The main cause of the gradient character of the transect is fresh water being sluiced from a canal into the Estuary (Fig. 1). The volume of this water varies throughout the year. The lowest discharges in the observation period with values of 1 150 000 $m^3 \cdot day^{-1}$ were found in July 1977, whereas the highest discharges with mean values of 2 250 000 $m^3 \cdot day^{-1}$ were measured in November 1977 (Netherlands Department of Public Works). The fresh water sluiced into the Estuary is heavily loaded with large amounts of organic matter in October, November, and December, when the potato flour mills are in operation. This polluted water contained about 200 mg organic $C \cdot l^{-1}$ and 1.5–3 mM of ammonium. During the rest of the year, when the discharged water is moderately polluted, these values were ≈ 50 mg $C \cdot l^{-1}$ and 0.1–0.5 mM of ammonium.

The dispersal of the canal water over the Estuary is irregular, and depends on tidal currents and winds. The transect stations in the vicinity of the sluice are, however, likely to be flushed more frequently with this highly polluted fresh water than

other transect stations. This assumption is supported by the observation of a salinity gradient on the mud flat (Table II).

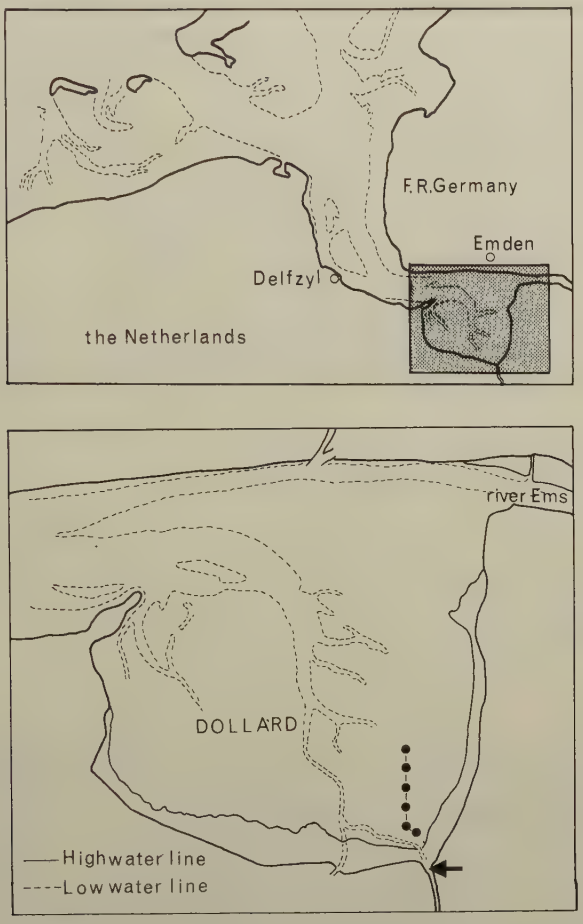


Fig. 1. Map of the Eems–Dollard Estuary, showing the transect (•—•) on the mud flat and the discharge point (→): the transect stations are located at distances of 0.8, 1.2, 1.7, 2.2, 2.7, and 3.2 km from the discharge point.

THE SPECIES COMPOSITION

The transect was sampled seven times during 1977 and 1978. The top layer (3–4 mm) of the sediment at five stations was scraped off and transferred to the laboratory. Using the lens tissue method (see Admiraal, 1977a) at least 0.5 l of a concentrated and relatively clean suspension of the epipelic diatom population was obtained per station. This suspension was used for: (1) the determination of species

composition, and (2) a series of experiments testing the capacity of the population to withstand selected environmental stresses (described below).

Permanent microscope slides were prepared from the diatom suspension after oxidation with hydrogen peroxide (with a few drops of sulphuric acid added) and washing in demineralized water. The frustules were embedded in highly refractive resin (Clearax). 200–300 cells per sample were identified on the basis of the works of Hustedt (1930) and Hendey (1964). The relative abundance of each species was expressed as a percentage occurrence in the total number of cells identified. Microscopic comparison of tissue-harvested diatom populations with living samples scraped directly from the sediment of the mud flat did not indicate that the lens-tissues selectively harvested some species and not others.

Dense mats of blue-green algae (mainly *Lyngbya martensiana* Menegh., pers. comm. W. T. Stam) and patches of *Euglena* sp. were found in summer 1977 and spring 1978 at the stations near the sluice. These algae were not considered in the present investigation and did not interfere with the present experiments on the diatom populations.

TOLERANCE TESTS ON NATURAL POPULATIONS

Three series of experiments were carried out on suspensions of natural diatom populations (harvested as described). The ranges of salinity, ammonia concentration, and sulphide concentration tolerated by the diatom samples were determined in these experiments.

Salinity tolerance

The rate of photosynthesis of suspended diatom samples was measured in a series of media with salinities of 0.7, 2.7, 4.7, 8.7, 16, and 34‰. These media were prepared by dilution of a stock solution of artificial sea water. The stock solution with a salinity of 33‰ contained per litre: NaCl, 24.5 g; $MgCl_2 \cdot 6\ H_2O$, 9.8 g; $KCl \cdot 2\ H_2O$, 0.53 g; $Na_2SO_4 \cdot 10\ H_2O$, 7.3 g, and K_2SO_4, 0.85 g. This solution was diluted with demineralized water to obtain a series of solutions with the following salinities: 0, 2.0, 4.0, 8.0, 15, and 33‰. $NaHCO_3$ (200 mg $\cdot$ l^{-1}) and Tris-buffer (500 mg $\cdot$ l^{-1}, pH 8.0) were added to all these media. These two additions account for a salinity of $\approx$0.7‰, hence the salinities of the test solutions became 0.7, 2.7, 4.7, 8.7, 16, and 34‰.

The diatom cells were transferred into 10-ml portions of the test solutions and were pre-incubated for 1 h at 12 °C and an irradiance of $\approx 10\ \mu E \cdot m^{-2} \cdot s^{-1}$. Then the rate of oxygen production of the suspended sample was measured at 12 °C and an irradiance of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ using a Clark-type oxygen probe (Admiraal, 1977a). From the oxygen production rates, measured for each diatom sample in the six test media, the salinity range was determined, in which the photosynthetic oxygen production rate of the sample was at least 75% of the highest rate measured.

This reduced photosynthetic rate was often reached at a salinity between those of the test solutions. In these cases the upper and lower level of salinity permitting 75% of the highest photosynthetic rate, was determined by interpolation. The salinity of the interstitial water of the top layer (3–4 mm) of the sediment was measured with an osmometer (Advance Instruments, Inc.).

Ammonia tolerance

The photosynthetic rate of diatom samples was determined in media with a salinity of 6‰ and concentrations of ammonia of 0, 1, 3, and 10 mM. These measurements were based on a previous study of inhibitory effects of ammonia (Admiraal, 1977b). In the present experiments the composition of the medium (except for the ammonia additions), the pre-incubation period, the temperature and the irradiance during the measurement of photosynthetic oxygen production were similar to those used for the measurements on salinity tolerance. From the series of measurements on each diatom sample the ammonia concentration was determined at which the photosynthetic oxygen production rate of the sample was 75% of the highest rate measured. This inhibitory concentration of ammonia was obtained by interpolation of the photosynthetic rates measured at ammonia concentrations of 1, 3, and 10 mM.

Sulphide tolerance

The tolerance of diatom samples to free sulphide was tested by measuring their survival in media with sodium sulphide during dark incubation in anoxic bottles (see Admiraal & Peletier, 1979b). The test conditions used in the present experiments were combinations of sulphide concentration (0, 1, 3, or 6 mM) and incubation time (5, 24 or 48 h). The photosynthetic rate of each diatom sample was measured after every period of exposure to solutions of sulphide and after reference incubations without sulphide. The ratio of photosynthetic rates, measured after exposure to sulphide and after a reference incubation, respectively, was used as a measure for the survival of the diatom sample in the sulphide solution. In a previous study (Admiraal & Peletier, 1979b) we showed that the degree of survival calculated from the photosynthetic rate agreed with microscopic observations of the number of diatom cells that survived exposure to sulphide.

BENTHIC COMMUNITIES UNDER EXPERIMENTAL CONDITIONS

Samples of surface sediment with their diatom populations were transferred to the laboratory and were incubated under a variety of environmental conditions. Comparable experiments on natural diatom populations were carried out in a previous study (Admiraal, 1978). The selective effect of environmental conditions on the species composition of diatom populations was investigated in the present experiments.

The top layer ($\approx$5 mm) of the sediment with diatoms was scraped from the mud flat (3.2 km from the sluice, Fig. 1) and transferred to the laboratory. The sediment and diatoms were mixed and spread out in seven Perspex boxes (measuring 20 × 20 cm or 15 × 15 cm) in layers 5 mm thick. These boxes were connected with a multichannel peristaltic pump, which pumps $\approx$4 l of artificial sea water medium per day through each system. This medium was enriched artificial sea water and was also suitable for growing diatoms in batch cultures. It was similar to that used for the experiments on salinity tolerance, except that the salinity was adjusted to 6‰. It was enriched as follows: $NaHCO_3$, 200 mg · l^{-1}; Tris-buffer, 500 mg · l^{-1} (pH 8.0); "minor" elements in half the concentrations given by Provasoli *et al.* (1957, p. 414); trace elements and vitamins in half the concentrations given for the "f" medium of Guillard & Ryther (1962); HBO_3, 13 mg · l^{-1}; $Na_2SiO_3 \cdot 9\ H_2O$, 100 μM; NaH_2PO_4, 18 μM; KNO_3, 500 μM. Nitrate was given as the sole source of nitrogen except for the system in which the influence of high ammonia concentrations was tested.

TABLE I

Sets of physico-chemical conditions tested for their selective influence on the species composition of mixed diatom populations kept in laboratory culture systems.

Conditions tested	Temperature (°C)	Quantum irradiance ($\mu E \cdot m^{-2} \cdot s^{-1}$)	Hours of light per day	Salinity (‰)	Concentration of ammonia (mM)
Set 1 (reference)	12	70	12	6	0
Set 2 (1‰)	12	70	12	1	0
Set 3 (20‰)	12	70	12	20	0
Set 4 (NH_3)	12	70	12	6	2.5
Set 5 ("winter")	4	70	4	6	0
Set 6 ("summer")	25–30 light period 20 dark period	700	16	6	0
Set 7 (dark)	4	0	0	6	0

Field samples were incubated under the seven different conditions shown by Table I. The pH of the medium in the systems increased up to 9 during the light period. Two independent experiments were carried out, the first one (referred to as I) started on 28 February 1978 and the second one (referred to as II) started on 13 March 1978. In the Experiments I and II the field samples were incubated for 4 and 5 days, respectively. After the incubation period, all systems were kept for 1 day at the conditions of the reference system. Then the systems were drained and descriptions of the diatom populations were made by the following methods.

(1) Two cores of 1.5 cm^2 each were suspended in 8 ml of medium. A drop of the suspension was transferred with a rod to a microscope counting chamber (Neubauer, improved; 0.1 mm deep). The diatom cells were counted at a magnifica-

tion of 200 × under a Zeiss photomicroscope using fluorescent light. The cell concentrations were calculated per mm^2 bottom surface of the experimental boxes.

(2) Two layers of lens tissue were spread out on the surface of the sediment; the upper one was used to obtain clean samples of the epipelic diatom population. This sample was used only for establishing the species composition of a fraction of closely related *Navicula* species that could not be identified using fluorescence microscopy. Diatom samples were identified as described before.

Results

DISTRIBUTION OF DIATOM SPECIES

The epipelic diatom populations on the transect were composed of a very limited number of species and the distribution showed spatial and temporal patterns. The seven species shown in Fig. 2 comprise more than 95% of the total number of identified cells. *Navicula pygmaea* Kützing, *N. salinarum* Grunow, and *Nitzschia* sp. were dominant species at stations near the sluice (Figs. 1 and 2), whereas *Navicula flanatica* Grunow, *N. phyllepta* Kützing, and *Gyrosigma fasciola* (Ehr.) Cleve were found mainly on stations at the other end of the transect (Fig. 2). *Navicula* cf. *cryptocephala* Kützing was found on all transect stations, but its abundance was variable.

There was a clear succession of species during the year of observation. *N. salinarum* was absent in June and August, whereas *Nitzschia* sp. was only present on these two occasions (Fig. 2). *Navicula flanatica* was only abundant in the spring of 1977 and 1978 and disappeared in the summer, *N. pygmaea* was a dominant species in the summer and autumn of 1977, but disappeared in winter. Its number increased again in spring. *Gyrosigma fasciola* and *Navicula* cf. *cryptocephala* were found throughout the year in variable numbers, the former being most abundant in November and December.

The succession of species was to some extent seasonal, regulated by temperature and irradiance, but it was possibly also related to the discharges of polluted fresh water. Table II shows that the extension of the area with superficial (<2 mm) black sediment layers is greatest in November and December, during peak discharges of waste water.

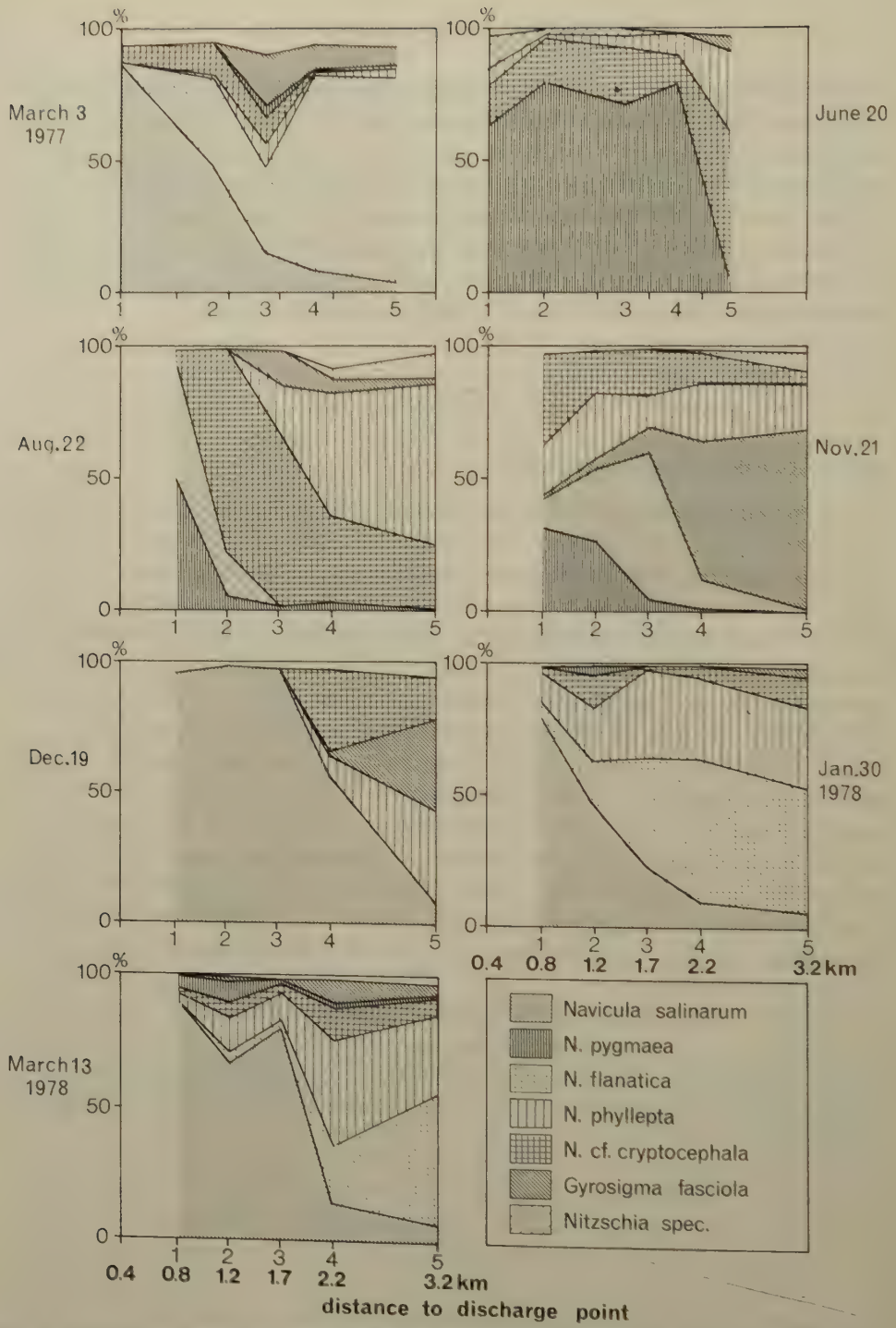


Fig. 2. Distribution of epipelic diatom species on the mud-flat transect during the observation period: the relative abundance of each species was expressed as a percentage of the total number of cells identified; the distance of the sampled transect stations to the discharge point is shown in km (see Fig. 1); the populations harvested are also numbered 1–5 and these numbers refer to experiments carried out with these populations (see Tables III, IV, and V).

SALINITY TOLERANCE

The salinity of the interstitial water, harvested from the top layer of sediment shows a gradual increase over the transect from ≈1‰ near the sluice to ≈6‰ at the other end (30 January and 13 March, Table II). The diatom populations collected from the transect were capable of high photosynthetic rates in media with a wide range of salinity (Table III). Most of the samples were hardly inhibited even at

TABLE II

Thickness of the aerobic top layer of the sediment covering the black sediment layer and measurements of the salinity of the interstitial water of the top 3 mm of sediment of the mud flat stations: the Station numbers 1–5 refer to Fig. 2; the enclosed part of the table shows the extension of the area with superficial black sediment layers during periods of large waste water discharge.

	Station numbers				
Sampling date	1	2	3	4	5
	Depth of aerobic top layer (mm)				
20 June 1977	1–2	1–2	2–3	2–3	5
22 Aug. 1977	1–2	1–2	2	5	10
21 Nov. 1977	0–1	0–1	1–2	1–2	2
19 Dec. 1977	1–2	1–2	1–2	1–2	5
30 Jan. 1978	1–2	1–2	1–2	2–4	2–4
13 March 1978	2	2–5	2	5	10
	Salinity of interstitial water (‰)				
30 Jan. 1978	1.5	2.4	2.7	3.6	5.0
13 March 1978	1.3	3.3	4.0	4.8	6.6

TABLE III

Salinity ranges (‰) of media in which the photosynthetic rate of diatom populations exceeded 75% of the highest rates, observed at optimum salinity; the lower and upper levels of this salinity range were obtained by interpolation of photosynthetic oxygen production rates, measured in media with salinities of 0.7, 2.7, 4.7, 8.7, 16, and 34‰; the species composition of the diatom samples is shown in Fig. 2; the Station numbers are those in Fig. 2; n.d., not determined.

	Station number				
Sampling date	1	2	3	4	5
20 June 1977	0.7–20	0.7–19	n.d.	0.7–19	0.7–21
22 Aug. 1977	0.7–14	0.7–21	0.7–22	2.3–20	1.5–26
21 Nov. 1977	0.7–19	0.7–20	0.7–21	2.5–22	2.4–23
19 Dec. 1977	0.7–19	0.7–15	1.6–17	0.7–17	1.9–19
30 Jan. 1978	0.7–20	0.7–23	0.7–22	0.7–24	0.7–25
13 March 1978	0.7–22	0.7–20	0.7–13	0.7–25	0.7–22

0.7‰ *S*. The observations on six samples, however, showed that a higher salinity was required for the diatom population to reach a photosynthetic rate of 75% of the highest rate at optimum salinity (Table III). The populations collected from Stations 4 and 5 on 22 August, required slightly higher salinities (≈2‰) to reach a high photosynthetic rate. At this sampling date the two populations from Stations 4 and 5 were dominated by *N. phyllepta*, indicating that the photosynthetic capacity of this species is negatively affected by salinities of <2‰ (Fig. 2). The observations on 21 November showed that populations dominated by *Gyrosigma fasciola* had a lower limit of tolerance at ≈2.5‰ *S* (Table III, Fig. 2). The population from Station 5, sampled on 19 December, showed large numbers of *G. fasciola* and *Navicula phyllepta* and also had a depressed photosynthetic rate at <1.9‰ *S*. A single observation on 19 December showed that the population at Station 3, consisting of a monoculture of *N. salinarum* (Fig. 2), had a lower salinity limit of 1.6‰. The photosynthetic rate of comparable monocultures of *N. salinarum*, obtained from Stations 1 and 2, sampled at the same time was, however, not affected by a salinity of 0.7‰. So, it seems very unlikely that *N. salinarum* is really inhibited by low salinities. Also populations with large numbers of *N. pygmaea*, *N. flanatica* and *N.* cf. *cryptocephala* are not inhibited by the lowest test concentration (Table III, Fig. 2). The highest salinities that permit a photosynthetic rate, reaching 75% of the rate measured at optimum salinity, were 14–26‰. There is no indication that diatom samples, collected near the sluice or collected during large freshwater discharge were more sensitive to these high salinities (Table III). The upper levels of salinity, at which rapid photosynthesis is still possible, are considerably higher than the highest salinity measured in the interstitial water of the mud flat (6.6‰, Table II).

AMMONIA TOLERANCE

Populations of diatoms, collected from the transect showed rather large differences in their capacity to maintain a high photosynthetic rate in the presence of

TABLE IV

Ammonia concentrations (mM) of media in which the photosynthetic rate of diatom populations was decreased to 75% of the highest rate, observed at lower or zero concentrations: the inhibitory ammonia concentrations were obtained by interpolation of photosynthetic oxygen production rates, measured at 0, 1, 3 and 10 mM of ammonia; the Station numbers are those in Fig. 2.

Sampling date	Station number				
	1	2	3	4	5
20 June 1977	5	6	6	5	5
22 Aug. 1977	6	6	6	2	2
21 Nov. 1977	8	9	9	7	8
19 Dec. 1977	8	10	7	>10	4
30 Jan. 1978	10	7	5	5	4
13 March 1978	9	6	7	3	3

ammonia concentrations of 1–10 mM (Table IV). During periods of large discharges of polluted fresh water with high ammonia concentrations, nearly all the populations on the transect tolerated ammonia concentrations of $\geqslant 7$ mM (Table IV). Comparison of the ammonia tolerance of the populations (Table IV) and the species composition of the populations (Fig. 2) show that ammonia concentrations of 7–10 mM were tolerated only by *N. salinarum* and *Gyrosigma fasciola*. Concentrations of 5–6 mM were tolerated by *Navicula pygmaeae* and *N.* cf. *cryptocephala* and 2–4 mM was the highest concentration in which a high photosynthetic rate is still possible in *N. phyllepta* and *N. flanatica*.

SULPHIDE TOLERANCE

Table V shows that diatom populations from transect Stations 1 and 2 near the discharge point were able to withstand a 48-h exposure to 3 mM of sulphide better at least than populations from the Station 5. The same distribution of sulphide-tolerant and more or less sulphide-sensitive populations on the transect was indicated by the results recorded after a 24-h exposure to 3 mM of sulphide. An incubation at 1 mM of sulphide during 48 h did not inhibit most of the populations tested (ratio 0.8–1.3, Table V), except for a few from Stations 4 and 5 (ratio 0.6–0.7, Table V). Concentrations of 6–7 mM of sulphide completely inhibited all the populations, when these were incubated for 24 h. The single series of experiments, in which the populations still showed photosynthetic activity, also indicate the highest tolerance of populations taken near the sluice. Short incubations of 5 h in these 6- to 7-mM solutions of sulphide permitted some photosynthetic activity but did not indicate clear differences in tolerance of populations from the Stations 1–5.

The populations, sampled on various occasions, reacted differently to similar exposures to sulphide. All populations sampled on 22 August, for example, showed no photosynthesis after exposure to 3 mM of sulphide for 48 h, whereas on other sampling dates the photosynthetic rate after a similar exposure was $\approx 50\%$ of the photosynthetic rate in the reference system. This observation can be explained by the abundance of a sensitive species in August (*N.* cf. *cryptocephala*, Fig. 2). It has, however, been shown by Admiraal & Peletier (1979b) that an independent series of measurements may show differences in sulphide tolerance for unknown reasons. For that reason sulphide tolerances should be compared only for diatom populations that were sampled and tested at the same time.

Comparison of the ratio, indicating sulphide tolerance (Table V), and the species composition of the same populations (Fig. 2) indicates that *N. salinarum* and *N. pygmaea* are relatively tolerant species. Probably *N. phyllepta* and *N. flanatica* are somewhat less tolerant. Only the samples containing considerable numbers of *Gyrosigma fasciola*, showed decreased photosynthetic rates after a 48-h exposure to 1 mM of sulphide. Hence *G. fasciola* is probably a relatively sensitive species.

TABLE V

Results of sulphide tolerance tests carried out on the same diatom samples as referred to in Tables III and IV, and in Fig. 2: the ratio of the photosynthetic rate, measured after incubation in sulphide-containing medium, to the photosynthetic rate, measured after a reference incubation, is presented for each sample as a measure of its sulphide tolerance; n.d., not determined.

Sulphide (mM)	Incubation time (h)	Sampling date	Ratio mg $O_2 \cdot h^{-1}$ (+ sulphide) to mg $O_2 \cdot h^{-1}$ (ref.)				
			Station number				
			1	2	3	4	5
3	48	20 June 1977	0.7	0.7	0.7	0.8	0.1[5]
		22 Aug. 1977	0.0	0.0	0.0	0.0	0.0
		21 Nov. 1977	0.6	0.5	0.5	0.1	0.0
		19 Dec. 1977	0.2	0.6	0.4	0.1	0.1
		30 Jan. 1978	0.2	0.3	0.1	0.1[5]	0.1
		13 March 1978	0.5	0.4[5]	0.4	0.3	0.1
3	24	20 June 1977	n.d.				
		22 Aug. 1977	0.0	0.1	0.2	0.0	0.1
		21 Nov. 1977	n.d.				
		19 Dec. 1977	0.6	0.8[5]	1.0	0.5	0.3
		30 Jan. 1978	0.6	0.6	0.4	0.5	0.4
		13 March 1978	0.7	0.7	0.5	0.7	0.3
1	48	20 June 1977	n.d.				
		22 Aug. 1977	n.d.				
		21 Nov. 1977	0.9	0.9	1.0	0.6	0.7
		19 Dec. 1977	1.0	0.8	0.9[5]	1.0	0.6
		30 Jan. 1978	0.9	0.7	0.8	0.8	1.0
		13 March 1978	1.1	1.0	0.9	1.3	0.8
6–7	24	20 June 1977	0.0	0.0	0.0	0.0	0.0
		22 Aug. 1977	n.d.				
		21 Nov. 1977	0.0	0.0	0.0	0.0	0.0
		19 Dec. 1977	0.0	0.1	0.0	0.0	0.0
		30 Jan. 1978	0.0	0.1	0.0	0.1	0.0
		13 March 1978	0.4	0.4	0.2	0.2	0.1
6–7	5	20 June 1977	0.1	0.1	0.2	0.1	0.2
		22 Aug. 1977	n.d.				
		21 Nov. 1977	0.1	0.1	0.1	0.0	0.0
		19 Dec. 1977	0.3	0.1	0.0[5]	0.2	0.1
		30 Jan. 1978	0.1	0.3	0.2	0.3	0.3
		13 March 1978	0.8	0.9	0.8	0.9	0.7

EFFECTS OF STRESS ON SPECIES COMPOSITION

Several physico-chemical conditions selectively affected the species composition of natural diatom populations incubated on their sediments in the laboratory (Table VI). The numbers of cells in the sediment was specified for three groups of diatoms: *G. fasciola*, *Navicula* spp. and *Nitzschia* spp. The relative abundance of the *Navicula* spp. was further analysed using slides of clean frustules, prepared from samples obtained by the lens-tissue technique.

TABLE VI

Selective influence of sets of physico-chemical conditions on the species composition of natural diatom populations, incubated on their natural sediment in the laboratory: for specification of the test conditions see Table I; I and II refer to independent experiments started on 28 February and 13 March, 1978, respectively.

	Number of cells per mm^2						Relative species composition of *Navicula* fraction (%)									
	G. fasc.		*Nav.* spp.		*Nitzs.* spp.		*N. flan.*		*N. sal.*		*N. crypt.*		*N. phyll.*		*N. pygm.*	
Test condition	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
6‰ *S* (ref.)	400	400	7300	15600			67	45	1	17	7	3	20	34	0.5	1.2
1‰ *S*	100	300	3400	15000			24	21	33	27	15	15	26	1	1	1.3
20‰ *S*	300	1000	6100	14400			74	49	5	20	8	5	12	25	0	0
NH_3	140	500	5000	10500			4	14	77	75	16	6	3	5	0.5	0.3
"Winter"	50	400	5000	20600			82	71	7	1	6	1	5	27	0	0.6
"Summer"	300	1000	3800	10800	35000	≈10000	15	0	69	96	4	2	11	2	0.7	0
Dark	300	600	4500	13900			83	88	2	2	9	1	4	8	0.4	0.4

Low salinity (1‰) promoted the development of *N. salinarum* and *N.* cf. *cryptocephala*, whereas the fraction of *N. flanatica* decreased. High salinity (20‰) did not cause changes in the species composition of the diatom populations in comparison to a reference system. High concentrations of ammonia affected the population strongly. *N. salinarum* became the dominant species, whereas the number of *N. flanatica* and *N. phyllepta* declined. *Gyrosigma fasciola* and *Navicula* cf. *cryptocephala* were indifferent in this experiment. "Winter" conditions (Table I) were not selective in comparison with the conditions of a reference system, whereas "summer" conditions (Table I) affected the species composition of the population. *N. salinarum* was strongly augmented, while the numbers of *N. flanatica* decreased and other species were more or less indifferent. In the diatom population originally composed mainly of *Gyrosigma fasciola* and *Navicula* spp. a dense population of *Nitzschia* spp. developed.

Discussion

The diatom species on the polluted mud flat studied were distributed in clear patterns, the few species present having fairly constant features of temporal and spatial distribution. Two main reasons may explain these observations. First, the diatom species flourishing on the mud flat clearly have different capacities to cope with the extreme conditions brought about by the organic pollution, the locally decreased salinity and the very long daily exposure to air. Secondly, the sheltered position within the Eems–Dollard Estuary restricts the suspension of diatom cells by tidal waters and hence the gradients in species composition are only occasionally disturbed.

The following environmental factors are unlikely to be responsible for the observed spatial distribution patterns of the diatom species. The transect stations were sited at about the same level on the mud flat and hence the immersion of the stations by tidal waters is about the same. The sediments on all the transect stations are very soft and silty. The concentrations of inorganic nutrients (phosphate, nitrogen compounds, and silicate) are probably in surplus of the requirements of these diatoms. As benthic diatom species from the Estuary seem to be quite tolerant of oxygen depletion (Admiraal & Peletier, 1979b), the oxygen depletion of the water (and perhaps of the sediment) caused by the polluted discharge is unlikely to be the direct reason for the distinct distribution of species over the transect. The partial desiccation of the sediment that is occasionally found on the three landward stations (Fig. 1) may, however, be the cause of an extreme salinity regime at these stations.

Throughout the investigation we observed and harvested dense diatom populations that looked healthy under the microscope and produced very high photosynthetic rates in the experiments. Therefore, the conditions on the transect stations are not so adverse that they prevent the development of dense diatom populations.

TABLE VII

Survey of data on distribution of diatom species in the field and experimental data on the different capacities of species dominating the diatom populations on the mud flat transect: +, positive; −, negative; ±, intermediate; n.d., not determined.

	N. sal.	*N. pyg.*	*N.* cf. *crypt.*	*N. flan.*	*N. phyll.*	*G. fasc.*	Reference
Salinities permitting abundance on the mud flat (‰)	<2–4	<2–4?	no preference	>2 4	>1–4	>4?	Table II, Fig. 2
Salinities permitting >75% of the highest photosynthetic rate (‰)	0.7–20	0.7–20	0.7–20	0.7 20	2–23	2.5 22	Table III
Salinities permitting good growth of cultures (‰)	2–45	n.d.	4–45	n.d.	n.d.	n.d.	unpubl. results
Influence of low salinity (1‰) on the abundance in natural communities	+	n.d.	+	−	± or −	−	Table VI
Abundant during waste water discharge in Nov. and Dec.	+	±	±	−	+	+	Fig. 2
Ammonia concentrations (mM) reducing the photosynthetic rate	7–10	5–6	5–6	2–4?	2–4	7–10	Table IV, Fig. 2
Ammonia concentrations (mM) permitting good growth of cultures	2.5–5	n.d.	1–2.5	n.d.	n.d.	n.d.	Admiraal, 1977b
Influence of 2.5 mM of ammonia on the abundance in natural communities	+	n.d.	±	−	−	±	Table VI
Abundant on sediment with superficial black layers	+	+	±	±	±	−	Fig. 2, Table II
Indication of sulphide tolerance of species, based on tolerance of field samples	+	+	n.d.	±	±	−	Table V, Fig. 2
Indication of sulphide tolerance of cultures	+	n.d.	±?	n.d.	n.d.	n.d.	Admiraal & Peletier (1979b)
Restricted to particular seasons	winter–spring	summer–autumn	no	spring, not in summer	no	no	Fig. 2
Effect of high temperatures and high irradiance on the abundance in natural communities	+	n.d.	±	−	− or ±	±	Table VI
Capacity of cultures to grow heterotrophically in the dark	+	n.d.	+	n.d.	n.d.	n.d.	Admiraal & Peletier (1979a)

Comparison of the information on natural distribution patterns of diatoms on the transect with the data from experiments (Table VII) elucidates a series of mechanisms determining the distribution patterns. Salinity proved to have a strong influence on mixed diatom populations kept on their natural sediments in the laboratory. This influence cannot be predicted in all cases from the optima obtained for diatom species in experiments testing the effect of salinity on photosynthetic rate or rate of cell division. On the mud flat certain species occupy only a limited part of their potential salinity range. *Navicula salinarum* predominates in the field at low salinities, but showed good growth in cultures at salinities of 2–45‰. Yet, only at the lower limit of its salt tolerance did it become a dominant species in selection experiments and in the field (Table VII). For other species (*Gyrosigma fasciola*, *Navicula phyllepta*) salinities <2‰ depressed the photosynthetic rate. Williams (1964) also found that the growth rate of *Gyrosigma fasciola* was decreased at salinities of 1–2‰. Thus the observations on *G. fasciola* show that the predominance of this species on the mud-flat stations far from the freshwater discharge point is caused by the inability of this species to grow at salinities of 1–2‰.

The observations on the influence of high ammonia concentrations on the photosynthetic rate, rate of division, and abundance of species (Table VII) support the conclusion that a high concentration of ammonia is a critical selective factor in the diatom populations of the mud flat. The two species capable of photosynthesis at ammonia concentrations of 7–10 mM (*Navicula salinarum* and *Gyrosigma fasciola*) bloomed during periods of wastewater discharge (November and December, Fig. 2). In agreement with this observation, the addition of ammonia to natural populations kept in the laboratory selectively promoted *Navicula salinarum*, while the cell numbers of *Gyrosigma fasciola* (and *Navicula* cf. *cryptocephala*) remained roughly the same. Since the rate of division of *Gyrosigma fasciola* is much lower than that of *Navicula salinarum* (current observ.) a strong increase in the cell numbers of the former would not be expected in these experiments. The abundance of *N. phyllepta* decreased only slowly during the discharge of polluted water (in November and December, Fig. 2) and this seems to contradict the low ammonia tolerance of this species shown in experiments (Table VII). The increase of *N. salinarum* closely parallels the observations of Van Raalte *et al.* (1976) on artificially fertilized plots of salt marsh. Addition of inorganic fertilizers and urea strongly promoted the dominance of *N. salinarum* within the plots. This was attributed to some "nitrogen-processing property" of *N. salinarum* which may be identical with ammonia tolerance.

Sullivan (1978) showed that the species composition of mud-flat diatom communities is related to ammonia concentrations in the sediment, ranging from 0.2 to 0.8 mM. The present observations and those of Sullivan (1978) and Van Raalte *et al.* (1976) show the importance of high ammonia concentrations as a selective factor in the benthic diatom populations. The ammonia concentrations tested in the present experiments were in the range of 1–10 mM, which is about the concentration in the polluted freshwater discharge. The tests were, however, carried out at pH 8.0 and

an irradiance of 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. It has been shown previously (Admiraal, 1977b) that pH ≈ 9 and an irradiance of $\approx 1000\ \mu E \cdot m^{-2} \cdot s^{-1}$ (as found in intertidal pools) greatly enhance ammonia toxicity. So in the field the ammonia concentrations inhibitory to photosynthesis may be much lower than those in the present experiments.

The data in Table VII support the hypothesis that well-developed black sediment layers near the surface of the mud flat promote the dominance of sulphide-tolerant species. A definite conclusion on the rôle of free sulphide in the selection of species, however, cannot be given for two reasons. First, we could not measure the concentration of free sulphide in the sediment, although the sulphur cycle of the mud flat is now being investigated by H. G. J. Schröder (pers. comm.). Preliminary measurements in the Eems–Dollard Estuary and results already published for marine sediments indicate highest concentrations of free sulphide of a few mM (for references see Admiraal & Peletier, 1979b). As a result the tolerance tests were also carried out with free sulphide concentrations in the mM-range. Secondly, the survival of diatoms in sulphide solutions can be different in independent experiments. The cause of the variations is unknown and was not excluded by rigorous standardization of procedures (Admiraal & Peletier, 1979b). These variations do not, however, obscure the observation that diatom populations, harvested from the most reduced sediments, have the highest sulphide tolerance.

High temperatures have been proposed by Hopkins (1964) as an important factor for several intertidal diatom species, restricting their occurrence in summer. The present observations on *N. flanatica* support this hypothesis (Table VII). Accordingly, *N. flanatica* was abundant in the field during spring 1977 and 1978, but it disappeared in summer 1977. The predominance of *N. flanatica* in spring populations was also observed in other parts of the Wadden Sea (Van den Hoek *et al.*, 1979). In contrast, *Nitzschia* spp. bloomed under experimental "summer" conditions (Table I) and one *Nitzschia* sp. (morphologically similar to one from the experiment) was found in the field in June and August only. Thus the seasonal distribution of two species can be explained by their specific response to high temperatures and high irradiance. The absence of *N. salinarum* in summer populations is being investigated.

Organic growth factors may affect the distribution of diatom species on the polluted mud flat. We have shown previously that 6 out of 10 diatom species from the Eems–Dollard Estuary (among them *N. salinarum*, *N.* cf. *cryptocephala* and *Nitzschia* cf. *thermalis*) were capable of heterotrophic growth in the dark (Admiraal & Peletier, 1979a). This capacity may be widespread among pennate diatom species (Lewin & Lewin, 1960; Hellebust & Lewin, 1977). The reduced conditions on the mud flat and the continuous supply of small organic molecules from the waste water may contribute to the dominance of the heterotrophically growing species *Navicula salinarum*, *N.* cf. *cryptocephala* and *Nitzschia* cf. *thermalis*. In the absence of such information on other dominant species from the mud flat, the importance of the effect of organic substrates in the distribution of species cannot yet be fully assessed.

The present study shows that the observed distribution patterns may be regulated, in the first place, by the capacities of the diatom species to cope with the extreme conditions in their habitat. But, in addition, interactions among diatom species seem to be important. The diatom populations on the polluted mud flat reach densities (of 2–400 mg chlorophyll $a \cdot m^{-2}$) that are among the highest in the Eems–Dollard Estuary (Van den Hoek *et al.*, 1979). Mixed populations on natural sediment showed only a very moderate increase in density during laboratory incubations with ample nutrients and light (Table VI). These results indicate that the diatom populations on the mud flat reach a density that hardly allows further increase of the cell numbers. Dominant diatoms must be able to divide relatively rapidly in these dense populations. Therefore, not only the salinity tolerance, temperature tolerance, etc. of a species determines its abundance, but also its capacity to outgrow other species in dense populations.

Acknowledgements

We would like to thank Ir. L. Bouwman, Drs. F. Colijn, Professor C. van den Hoek, Professor H. Veldkamp, and Dr. P. de Wolf for discussion and for their critical comments on the manuscript.

References

Admiraal, W., 1977a. Salinity tolerance of benthic estuarine diatoms as tested with a rapid polarographic measurement of photosynthesis. *Mar. Biol.*, Vol. 39, pp. 11–19.

Admiraal, W., 1977b. Tolerance of estuarine benthic diatoms to high concentrations of ammonia, nitrite ion, nitrate ion and orthophosphate. *Mar. Biol.*, Vol. 43, pp. 307–315.

Admiraal, W., 1978. Experiments with mixed populations of benthic estuarine diatoms in laboratory micro-ecosystems. *Bot. Mar.*, Vol. 20, pp. 479–485.

Admiraal, W. & H. Peletier, 1979a. Influence of organic compounds and light limitation on the growth rate of estuarine benthic diatoms. *Br. phycol. J.*, Vol. 14, pp. 197–206.

Admiraal, W. & H. Peletier, 1979b. Sulphide tolerance of benthic diatoms in relation to their distribution in an estuary. *Br. phycol. J.*, Vol. 14, pp. 185–196.

Guillard, R. R. L. & J. H. Ryther, 1962. Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol.*, Vol. 8, pp. 229–239.

Hellebust, J. A. & J. Lewin, 1977. Heterotrophic nutrition. In, *The biology of diatoms*, edited by D. Werner, Blackwell, Oxford, pp. 169–197.

Hendey, N. I., 1964. *An introductory account of the smaller algae of British waters, part V: Bacillariophyceae (diatoms)*. Fisheries Investigations Series IV, Her Majesty's Stationery Office, London, 317 pp.

Hopkins, J. T., 1964. A study of the diatoms of the Ouse estuary, Sussex. III. The seasonal variation in the littoral epiphyte flora and the shore plankton. *J. mar. biol. Ass. U.K.*, Vol. 44, pp. 613–644.

Hustedt, F., 1930. Bacillariophyta (Diatomeae). In, *Die Süsswasserflora Mitteleuropas*, edited by A. Pascher, Fisher Verlag, Jena, Heft 10, 466 pp.

Lewin, J. C. & R. A. Lewin, 1960. Auxotrophy and heterotrophy in marine littoral diatoms. *Can. J. Microbiol.*, Vol. 6, pp. 127–133.

Provasoli, L., J. J. A. McLaughlin & M. R. Droop, 1957. The development of artificial media for marine algae. *Arch. Mikrobiol.*, Vol. 25, pp. 392–428.

SULLIVAN, M. J., 1978. Diatom community structure: taxonomical and statistical analysis of a Mississippi salt marsh. *J. Phycol.*, Vol. 14, pp. 468–475.

VAN DEN HOEK, C., W. ADMIRAAL, F. COLIJN & V. N. DE JONGE, 1979. The role of algae and seagrasses in the ecosystem of the Wadden Sea: a review. In, *Flora and vegetation of the Wadden Sea*, edited by W. J. Wolff. Report 3 of the Wadden Sea Working Group, pp. 9–118.

VAN RAALTE, C. D., I. VALIELA & J. M. TEAL, 1976. The effect of fertilization on the species composition of salt marsh diatoms. *Water Res.*, Vol. 10, pp. 1–4.

WILLIAMS, R. B., 1964. Division rates of salt-marsh diatoms in relation to salinity and cell size. *Ecology*, Vol. 45, pp. 877–886.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 177–196
© Elsevier/North-Holland Biomedical Press

GEOGRAPHIC PATTERNS OF CORAL BIOEROSION: A PRODUCTIVITY HYPOTHESIS

RAYMOND C. HIGHSMITH[1]

Department of Zoology, University of Washington, Seattle, WA 98195, U.S.A.

Abstract: The numbers of boring bivalves in corals in large museum collections were used to indicate relative bioerosional damage to the corals. The proportion of corals from different locations that contain boring bivalves is highly correlated with global patterns of plankton primary productivity. The densities of five other, non-boring, groups of planktivores associated with the same corals are similarly correlated with productivity.

The proportion of corals containing boring bivalves and the number of boring bivalves per coral head can be ranked by region as follows: eastern Pacific > western Atlantic > Indian Ocean > western Pacific. This ranking also corresponds to primary productivity differences.

Measurements of the basal, margin of live tissue, and maximum circumferences of the coral heads indicate that western Atlantic massive corals have more of their skeletal surface exposed to borers, i.e. not covered by live tissue, than do Indo-Pacific corals. Consequently, the former also have weaker basal attachments which suggests that they are more likely to be dislodged during storms. The reason why massive corals in the western Atlantic tend to have less of their skeleton covered by live tissue than corals in the rest of the world is unknown.

INTRODUCTION AND HYPOTHESIS

Recently, X-radiographic techniques have been developed that make it possible to quantify accurately damage to coral skeletons by boring organisms. The mean amount of skeletal material excavated from massive corals in Florida (Hein & Risk, 1975) and Barbados (MacGeachy & Stearn, 1976) is 28% and 15%, respectively. In the Pacific, massive corals at Enewetak average less than 4% excavation (Highsmith, 1979). Boring sponges were responsible for the most skeletal damage in all three studies. Boring bivalves accounted for $\approx 15\%$ of the excavation in Florida corals and the mean number of bivalves per head at Barbados was 3.1 At Enewetak, only three bivalves were found in 102 heads.

These regional differences, i.e. larger boring sponges and higher boring bivalve densities in the western Atlantic than in the Pacific, raise the possibility of a fundamental difference between Caribbean and Indo-west Pacific coral reefs additional to those listed by Milliman (1973). Bertram (1936), however, estimated that boring bivalves were responsible for colony detachment in 20% of cases at Al-Ghardaqa, Red Sea. Relatively high densities of boring bivalves also occur in corals at Phuket, Thailand (Nielsen, 1976) and Heron Island, Australia (Connell, 1973; Kleemann,

[1] Present address: Smithsonian Tropical Research Institute, Box 2072, Balboa, Republic of Panama.

1977). Conversely, corals at Curacao, in the western Atlantic, are only 1–2% excavated (Bak, 1976). Thus, it is apparent that a simple inter-ocean explanation for bioerosional differences is inadequate. Because sponges and bivalves are primarily filter-feeders, I hypothesize a direct relationship between the abundance of these boring organisms and plankton primary productivity in different geographic regions. This paper reports a test of this hypothesis based on examination of several hundred corals from a variety of geographic locations.

Methods

Massive corals were examined at the Smithsonian Institution, the Museum of Comparative Zoology at Harvard University, and at the Peabody Museum of Natural History at Yale. Since I was not at liberty to section the corals and measure the actual volume excavated, I counted boring bivalve siphonal openings present in each head. These openings are sufficiently characteristic to permit easy recognition of the genus of bivalve responsible (MacGeachy & Stearn, 1976). From analysis of the results of Hein & Risk (1975), MacGeachy & Stearn (1976), and my observations at Enewetak, I showed that the abundance of boring bivalves tends to fluctuate directly with overall bioerosional damage to corals (Highsmith, 1979). Therefore, abundances of boring bivalves are useful estimates of relative bioerosional damage to corals from different geographic locations. I also recorded abundances of non-boring bivalves, ahermatypic corals, serpulid polychaetes, and barnacles attached to the coral skeletons.

Most boring organisms settle on and bore into dead surface areas around the base of corals (Bak, 1976; MacGeachy & Stearn, 1976; Highsmith, 1979). The ratio of the basal attachment circumference to the live tissue margin circumference is a convenient estimate of the extent to which basal surfaces of massive corals are protected from attack by borers, i.e. covered by live tissue (Highsmith, 1979). A ratio of one indicates the base is covered with live tissue down to the substratum; lower ratios reflect increasing amounts of dead surface exposed to attack. Similarly, the ratio of the circumference of the basal attachment to maximum head circumference indicates relative strength of attachment to the substratum (Highsmith, 1979); the smaller the ratio of base to maximum circumference, the lower the strength of attachment.

Plankton primary productivity at different geographic locations was determined by reference to the Koblentz-Mishke *et al.* (1970) map of primary production in the world ocean (Fig. 1).

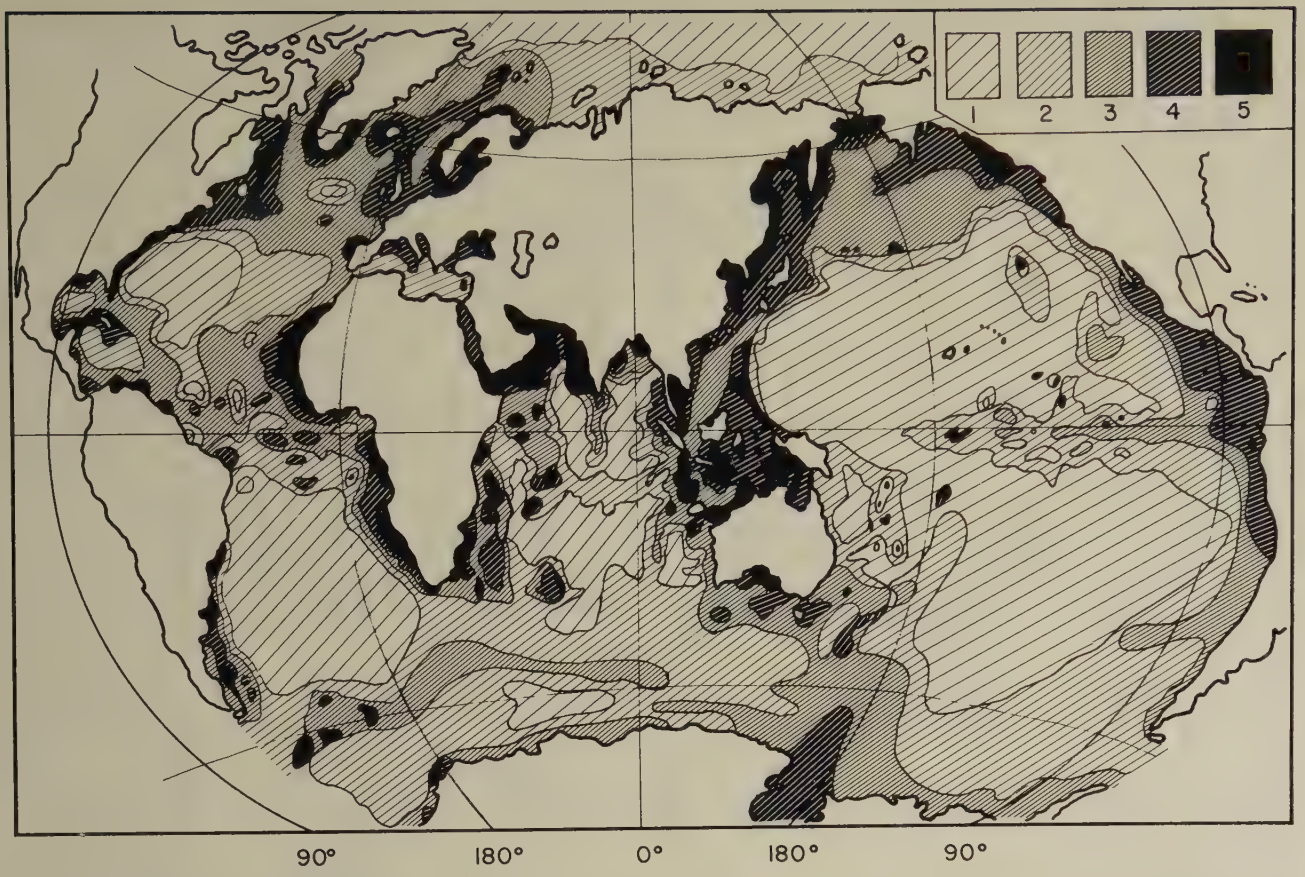


Fig. 1. Distribution of gross primary production in the world ocean: units are in mg $C \cdot m^{-2} \cdot day^{-1}$; 1, <100; 2, 100–150; 3, 150 250; 4, 250–500; 5, >500; reproduced from Koblentz-Mishke *et al.* (1970), p. 185, with permission of the National Academy of Sciences, Washington, D.C.

RESULTS

GEOGRAPHIC DATA AND SKELETAL FACTORS

The proportions of heads with boring bivalves and mean number of boring bivalves per coral head (Table I) differ in the four oceanic areas considered. For percentage of massive corals containing boring bivalves, the areas rank as follows: eastern Pacific > western Atlantic > Indian Ocean > western Pacific (Fig. 2). All dif-

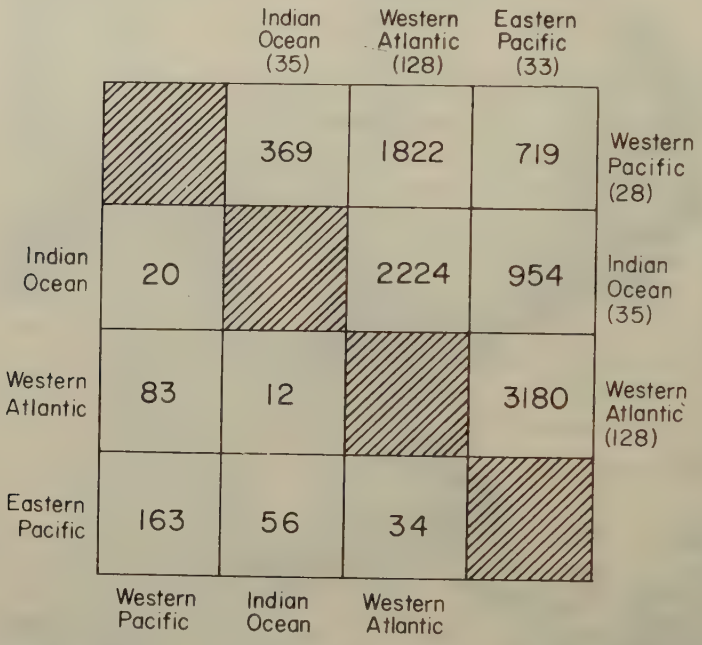


Fig. 2. Lower left, χ^2 values for 2 × 2 contingency tests on data in Table I; ocean regions in rows have a higher proportion of corals with boring bivalves than regions in the corresponding column; all values are significant at $P < 0.0005$. Upper right, J values for Jonckheere's test for ordered alternatives (Hollander & Wolfe, 1973) for coral heads containing one or more bivalves; ocean regions in columns have more bivalves per head than regions in corresponding rows; number of coral heads with boring bivalves is indicated in parentheses.

ferences are significant at $P < 0.0005$ (2 × 2 chi-square tests). Considering only corals with one or more boring bivalves, the number of bivalves per head is significantly different in each area and in the same rank order as above (Fig. 2; Jonckheere's test for ordered alternatives, $Z = 3.17$, $P = 0.0008$). In all four areas the mean number of boring bivalves and percentage of heads with boring bivalves are usually relatively high at locations near continents (Table I).

Most boring bivalves occur in dead surface areas on coral heads (Table I). After adjustment for individuals that initially penetrated a dead surface and were sub-

TABLE I

Mean number of boring bivalves per coral head and percentage of coral heads with boring bivalves: figures in parentheses are adjusted to show numbers of bivalves apparently encircled by coral growth after settlement which were removed from the live surface category and added to the dead surface column; live surface is that part of the skeleton covered by live coral tissue; the balance of the skeletal surface is counted as dead surface.

Location	Genus	Mean no. bivalves/head			No. of heads	% Heads with bivalves	Coral diameter range (cm)
		Total	Live surface	Dead surface			
Indian Ocean							
Red Sea	*Porites*	0.6	0	0.6	16	31	4–32
	Favia	0.3	0	0.3	10	30	7–26
	Goniastrea	4.4	0	4.4	9	67	11–26
Ceylon	*Porites*	0	0	0	2	0	5–7
	Favites	1.0	0	1.0	1	100	8
	Goniastrea	0	0	0	1	0	9
Singapore	*Favia*	1.4	0.6	0.8	10	60	7–13
	Goniastrea	1.7	0	1.7	3	67	7–8
	Platygyra	2.0	0.3 (0)	1.7 (2.0)	3	100	9–15
Maldive Islands	*Porites*	0.1	0	0.1	13	8	7–20
	Favia and *Favites (4)*	0.14	0.14 (0.07)	0 (0.07)	14	14	5–18
	Goniastrea	0	0	0	4	0	9–16
Seychelles Islands	*Porites*	0	0	0	2	0	8–9
	Favia	0	0	0	1	0	14
	Goniastrea	0	0	0	7	0	8–13
Chagos Islands	*Porites*	0	0	0	8	0	5–17
Zanzibar	*Porites*	0	0	0	2	0	3–5
Aldabra	*Porites*	6.6	5.6 (0.2)	1.0 (6.4)	5	80	11–14
	Goniastrea	0.5	0	0.5	2	50	12–17
Cosmoledo	*Porites*	0	0	0	1	0	6
	Goniastrea	0	0	0	2	0	11–12
Farquhar	*Favia*	0	0	0	2	0	5–7
Mauritius	*Porites*	0	0	0	8	0	9–21
	Favia	0	0	0	2	0	6
Totals					128	26	
Western Pacific							
Philippine Is.	*Porites*	0.6	0.13 (0)	0.5 (0.6)	8	50	9–15
	Favia	0	0	0	9	0	8–18
	Goniastrea	1.0	0.5	0.5	2	50	12–15
Saipan	*Porites*	0	0	0	14	0	5–17
	Favia	0	0	0	5	0	4–11
	Goniastrea	0	0	0	26	0	8–22
Enewetak*	*Porites*	0.02	0	0.02	63	1.6	6–32
	Favia	0	0	0	17	0	8–30
	Goniastrea	0.09	0	0.09	22	9	6–26
Gilbert Islands	*Porites*	0.5	0.2 (0)	0.3 (0.5)	32	9	4–41
	Favia	0	0	0	12	0	12–27
	Goniastrea	0	0	0	4	0	7–12

TABLE I *(continued)*

Location	Genus	Mean no. bivalves/head			No. of heads	% Heads with bivalves	Coral diameter range (cm)
		Total	Live surface	Dead surface			
Australia	*Porites*	0.1	0	0.1	13	8	9–41
	Favia	1.2	0	1.2	19	32	7–37
	Goniastrea	0.4	0.1	0.3	12	25	6–31
Samoa	*Porites*	0	0	0	10	0	8–15
Tonga	*Porites*	3.2	2.7 (0.6)	0.5 (2.6)	13	62	8–13
Tuamotu Islands	*Porites*	0.14	0.07 (0)	0.07 (0.14)	14	7	5–16
	Favia	0.09	0	0.04	11	9	5–16
Totals					306	10	
Eastern Pacific							
Gulf of Panama	*Porites*	33.7	18.7 (14.0)	16.5 (21.2)	11	100	6–19
	Pavona	20.9	5.1 (4.4)	15.8 (16.5)	11	91	5–18
Galapagos Is.	*Porites*	13.8	12.3	1.5	4	100	6–12
	Pavona	31.0	4.9 (4.6)	26.1 (26.4)	8	100	6–13
Totals					34	97	
Western Atlantic							
Florida	*Porites*	1.2	0.17	1.04	23	39	10–30
	Dichocoenia	1.8	0	1.8	5	60	8–36
	Colpophyllia	0	0	0	3	0	13–24
	Diploria	5.1	0.2 (0)	4.9 (5.1)	9	100	19–46
	Montastrea	3.2	0.1 (0)	3.1 (3.2)	10	50	11–31
Bahama Islands	*Porites*	1.3	0.3 (0)	1.0 (1.3)	15	40	11–27
	Dichocoenia	7.1	0.4 (0)	6.7 (7.1)	14	93	5–19
	Colpophyllia	1.0	0	1.0	2	100	9–18
	Montastrea	1.3	0.05 (0)	1.24 (1.3)	17	65	8–19
Puerto Rico	*Porites*	0.06	0	0.06	15	6	11–23
	Diploria	0.17	0	0.17	6	17	15–32
	Montastrea	1.0	0.17	0.83	6	33	6–43
Jamaica	*Porites*	0.09	0	0.09	11	9	7–18
	Diploria	0.3	0	0.3	3	33	8–18
	Montastrea	0	0	0	3	0	8–19
Haiti	*Porites*	0.14	0	0.14	7	14	7–15
	Diploria	0	0	0	1	0	18
St. Thomas	*Porites*	0.6	0.33	0.25	12	25	9–22
	Colpophyllia	0	0	0	2	0	9–25
	Montastrea	0	0	0	6	0	6–19
Belize	*Colpophyllia*	0	0	0	2	0	11–26
	Siderastrea	0	0	0	2	0	9–17
Guadaloupe	*Porites*	2.0	0	2.0	2	50	11–29

TABLE I *(continued)*

Location	Genus	Mean no. bivalves/head			No. of heads	% Heads with bivalves	Coral diameter range (cm)
		Total	Live surface	Dead surface			
Barbados	*Porites*	0.1	0	0.1	10	10	7–20
	Dichocoenia	4	0	4	1	100	8
Curacao	*Porites*	1.5	1.0 (0.6)	0.5 (0.9)	9	40	6–13
	Colpophyllia	0	0	0	7	0	10–21
Colombia	*Porites*	0	0	0	4	0	6–15
Panama	*Colpophyllia*	1.75	0.75	1.0	4	50	13–22
Venzuela	*Porites*	2.0	0.4 (0.2)	1.6 (1.8)	8	50	7–16
	Dichocoenia	20.0	0.3 (0)	19.7 (20)	3	67	9–24
	Diploria	3.4	0.4 (0)	3.0 (3.4)	7	57	8–22
	Montastrea	4.0	0.3 (0)	3.7 (4.0)	10	70	7–22
	Siderastrea	4.9	3.0 (1.1)	1.9 (3.8)	7	100	4–16
	Meandrina	5.3	0	5.3	3	100	21–25
	Stephanocoenia	3.9	2.7 (1.1)	1.2 (2.8)	11	91	6–17
Brazil	*Porites* sp.	0.9	0.5 (0.2)	0.4 (0.7)	12	42	7–25
	Favia sp.	1.2	0.4 (0.1)	0.8 (1.1)	10	70	5–11
Totals					282	45	

* Data are from a study of bioerosion at Enewetak (Highsmith, 1979).

sequently surrounded by coral growth, 79% of the bivalves were in dead surface zones. In all geographic areas excepting the eastern Pacific, 92% of bivalves occurred in dead zones. Some or all of the remainder may have been encircled by later coral growth but this could not be determined with certainty. Few boring bivalves are known to settle on live coral tissue (Highsmith, 1979).

Ratios of the circumference of the basal attachment to the circumference of live coral tissue were determined for locations in which five or more coral heads were measured (Table II). Ratios for western Atlantic locations are significantly lower than those for western Pacific ($P < 0.001$) or Indian Ocean locations ($P < 0.01$; Mann–Whitney U-tests). Western Pacific and Indian Ocean ratios are not significantly different. Data from the two eastern Pacific locations are not sufficient for statistical comparisons, but the values are closer to those for the Indian Ocean and western Pacific than those for the western Atlantic.

One consequence of failing to maintain tissue over the lower part of the skeleton

is that corals cannot enlarge their bases as they grow. As expected from the base/live ratios, the base to maximum circumference ratios (Table II) for the western Atlantic are lower than those for western Pacific ($P < 0.001$) or Indian Ocean sites ($P < 0.01$; Mann–Whitney U-tests). Western Pacific and Indian Ocean ratios are not significantly different.

TABLE II

Mean ratios of basal circumference to live tissue circumference and maximum circumference: N, number of coral heads measured; ratios were not calculated for samples of $N < 5$.

Location	N	Circumference ratios	
		Base/live	Base/max.
Indian Ocean			
Red Sea	34	0.61	0.65
Singapore	8	0.62	0.58
Maldive Is.	30	0.58	0.54
Seychelles Is.	9	0.45	0.40
Chagos Is.	8	0.85	0.78
Aldabra	5	0.65	0.52
Cosmol. & Farquhar	5	0.41	0.34
Mauritius	8	0.66	0.45
$\bar{X}$		0.60	0.53
Western Pacific			
Philippine Is.	17	0.76	0.65
Saipan	26	0.62	0.57
Enewetak	47	0.70	0.69
Gilbert Is.	29	0.63	0.55
Australia	34	0.45	0.40
Samoa	6	0.78	0.43
Tonga	12	0.72	0.55
Tuamotu Is.	24	0.72	0.59
$\bar{X}$		0.67	0.55
Eastern Pacific			
Gulf of Panama	12	0.62	0.61
Galapagos Is.	5	0.72	0.72
$\bar{X}$		0.67	0.67
Western Atlantic			
Florida	45	0.39	0.36
Bahama Is.	47	0.49	0.43
Puerto Rico	24	0.35	0.32
Jamaica	16	0.39	0.35
Haiti	7	0.40	0.39
St. Thomas	20	0.45	0.39
Barbados	6	0.52	0.48
Curacao	16	0.43	0.35
Venezuela	48	0.39	0.35
Brazil	22	0.35	0.33
$\bar{X}$		0.42	0.38

Although some of the mean ratios for geographic areas are not statistically different, they are in the same rank order, except for the eastern Pacific, as the percentage of corals with boring bivalves and mean number of boring bivalves per head. The eastern Pacific appears to be unique in that many bivalves occur in the live part of corals (Table I). Thus, eastern Pacific corals contain more boring bivalves than expected from the relatively high base/live circumference ratios (Table II).

Most western Atlantic massive coral genera do not occur in the Indo-Pacific. Members of the genus *Favia*, however, are closely related to *Montastrea* species (J. W. Wells, pers. comm.) and *Porites* is circumtropical. Therefore, I have compared western Atlantic and Indo-Pacific attachment/live ratios within *Porites* and between *Montastrea* and *Favia* (Table III). *Porites* attachment/live circumference ratios are significantly larger in the Western Pacific ($P < 0.001$) and Indian Ocean ($P < 0.001$) than in the western Atlantic (Mann–Whitney U-tests). There is no difference between

TABLE III

Mean ratios of basal circumference to live tissue circumference for massive *Porites* spp. and *Montastrea–Favia* spp. from different geographic locations: N, in parentheses, number of coral heads measured; ratios were not calculated for $N < 5$.

Location	*Porites* sp. (N)	*Montastrea–Favia* sp. (N)
Indian Ocean		
Red Sea	0.91 (16)	0.55 (9)
Maldive Is.	0.75 (13)	0.65 (10)
Chagos Is.	0.85 (8)	–
Mauritius	0.68 (6)	–
$\bar{X}$	0.81	
Western Pacific		
Philippine Is.	0.93 (6)	0.68 (9)
Saipan	0.76 (12)	0.94 (5)
Enewetak	0.70 (14)	0.78 (16)
Gilbert Is.	0.75 (17)	0.47 (9)
Australia	0.56 (9)	0.55 (16)
Samoa	0.78 (6)	–
Tonga	0.72 (12)	–
Tuamotu Is.	0.68 (13)	0.78 (11)
$\bar{X}$	0.71	0.68
Western Atlantic		
Florida	0.30 (20)	0.44 (9)
Bahama Is.	0.38 (14)	0.53 (17)
Puerto Rico	0.27 (15)	0.59 (6)
Jamaica	0.35 (10)	–
Haiti	0.38 (6)	–
St. Thomas	0.41 (12)	0.72 (6)
Barbados	0.49 (5)	–
Curacao	0.54 (9)	–
Venezuela	0.37 (9)	0.46 (10)
Brazil	0.27 (11)	0.47 (10)
$\bar{X}$	0.36	0.52

Western Pacific and Indian Ocean *Porites* ratios. For *Montastrea-Favia*, attachment/live ratios are also greater in the western Pacific than the western Atlantic ($P < 0.05$). Thus, the tendency for the basal area of western Atlantic massive coral skeletons to lack living tissues does not appear to be a growth characteristic of coral genera that happen to be restricted to that region.

BIOEROSION AND PRODUCTIVITY

Table IV gives the locations where corals were collected and the percentages of coral heads containing boring bivalves, arranged by productivity class (from Fig. 1). I used intermediate categories (100, 150, and 250 mg $C \cdot m^{-2} \cdot day^{-1}$) for locations on borders between productivity classes. The overall percentage of corals with boring bivalves for each class is strongly correlated with productivity (Fig. 3; Spearman rank

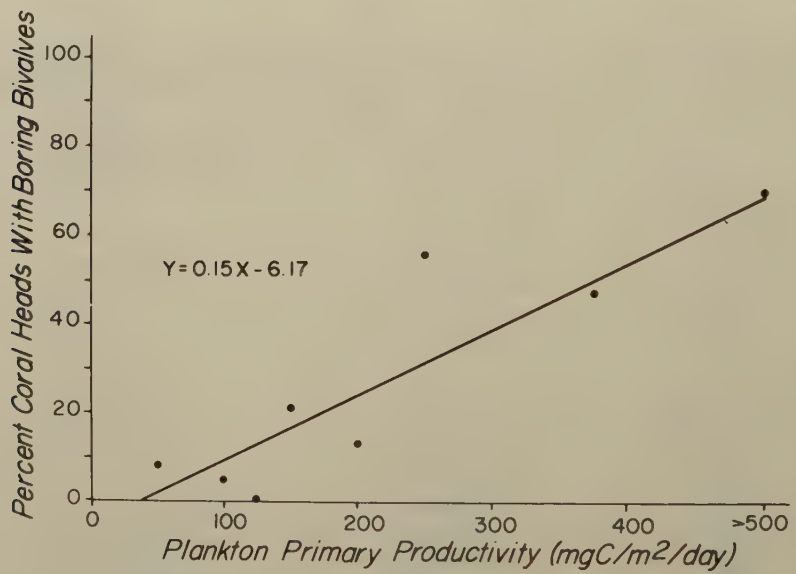


Fig. 3. Percentage of massive corals with boring bivalves as a function of plankton primary productivity: see Table IV for number of observations.

correlation coefficient, $r_s = 0.86$, $P < 0.01$). The regression $Y = 0.15\ X - 6.17$, where Y = percentage of corals with boring bivalves and X = gross primary productivity in mg $C \cdot m^{-2} \cdot day^{-1}$, accounts for 80% of the variance, and the regression coefficient is significant at the 0.005 level.

Abundances of non-boring bivalves ($r_s = 0.83$, $P < 0.01$), ahermatypic corals ($r_s = 0.87$, $P < 0.01$), and serpulid polychaetes ($r_s = 0.76$, $P < 0.05$) associated with the same coral heads are also all significantly correlated with phytoplankton productivity (Fig. 4A). Further, numbers of pyrgomatine (Balanidae: Pyrgomatinae) barnacles

TABLE IV

Location and percentage of coral heads with boring bivalves arranged according to phytoplankton productivity at the location (data from Fig. 1): *N*, in parentheses, number of heads.

Plankton primary productivity (mg C · m^{-2} · day^{-1})															
< 100		≈ 100		100–150		≈ 150		150 250		≈ 250		250–500		< 500	
Location (*N*)	%	Location (*N*)	%	Location (*N*)	%	Location (*N*)	%	Location (*N*)	%	Location (*N*)	%	Location (*N*)	%	Location (*N*)	%
Chagos Is. (8)	0	Gilbert Is. (48)	6	Seychelle Is. (10)	0	Maldive Is. (31)	10	Ceylon (4)	25	Bahama Is. (48)	67	Red Sea (35)	40	Aldabra (7)	71
Mauritius (10)	0	Samoa (10)	0	Farquhar (2)	0	Australia (44)	23	Zanzibar (2)	0	Curacao (16)	25	Philippine Is. (19)	26	Singapore (16)	69
Saipan (45)	0					Tonga Is. (13)	62	Cosmoledo (3)	0			Galapagos Is. (12)	100	Gulf of	
Enewetak (102)	3					Jamaica (17)	12	Puerto Rico (27)	15			Panama (4)	50	Panama (22)	95
Tuamotu Is. (25)	8					Haiti (8)	12	Belize (4)	0					Florida (50)	52
Brazil (22)	55					St. Thomas (20)	15	Barbados (11)	18					Venezuela (49)	76
						Guadaloupe (2)	50	Colombia (4)	0						
$\Sigma N = 212$		$\Sigma N = 58$		$\Sigma N = 12$		$\Sigma N = 135$		$\Sigma N = 55$		$\Sigma N = 64$		$\Sigma N = 70$		$\Sigma N = 144$	

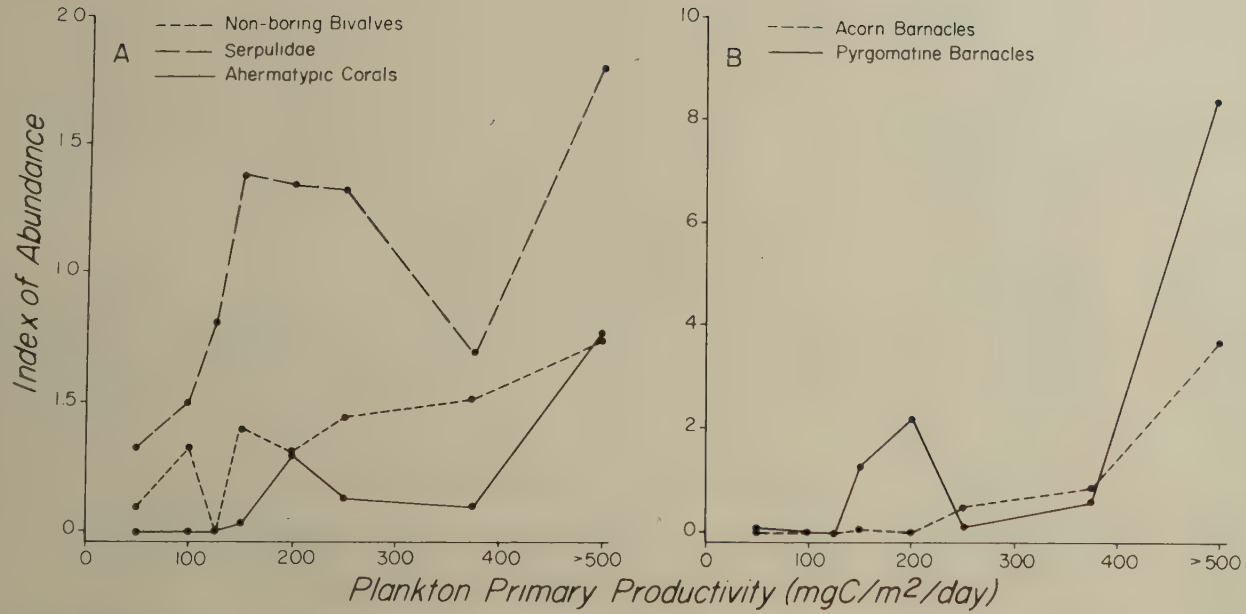


Fig. 4. A, abundance of serpulid polychaetes, non-boring bivalves, and ahermatypic corals attached to massive corals: index = mean number of individuals per head for non-boring bivalves and ahermatypic corals; for serpulids, index = mean rank where 1 = 1-4, 2 = few, 3 = several, 4 = many, 5 = numerous; B, mean number of pyrgomatine barnacles per coral head on live (at time of collection) surfaces and mean number of acorn barnacles per coral head on dead skeletal surfaces; see Table IV for number of observations.

($r_s = 0.73$, $P < 0.05$) in the live (at time of collection) portion of coral heads and acorn (Balanidae: Balaninae) barnacles ($r_s = 0.73$, $P < 0.05$) on dead surfaces are also significantly correlated with productivity (Fig. 4B). Data for Brazilian corals are excluded from the pyrgomatine curve in Fig. 4B. The 22 heads contained 2965 of these barnacles. The corals were collected near Porto Seguro, which according to Fig. 1 is in the lowest productivity category. This low ranking is probably due to a lack of data rather than low productivity (K. Sebens, pers. comm.). All other coastal zones in Fig. 1 are in higher productivity classes.

Discussion

BIOEROSION AND PRODUCTIVITY

The productivity map of Koblentz-Mishke *et al.* (1970) is based on data from over 7000 stations. These stations were spread over an enormous area and most were undoubtedly in open water. There have been very few studies of plankton primary productivity on coral reefs. Furthermore, most reef data were collected by different methods in different seasons and years, making valid comparisons difficult. The data available suggest that plankton productivity may, however, be seasonal in the western Atlantic (Curl, 1960; Richards, 1960; Jones, 1963; Margalef, 1968; Milliman, 1969; Reiswig, 1971; Glynn, 1973; Sander & Steven, 1973; Kidd & Sander, 1979) and Indian Ocean (Kabanova, 1968; Qasim & Sankaranarayanan, 1970; Qasim *et al.*, 1972; Krey & Babenerd, 1976), that plankton productivity on western Atlantic reefs is generally higher than that on western Pacific reefs (Doty & Oguri, 1956; Marshall, 1965; Jeffrey, 1968; Gordon *et al.*, 1971; Marshall *et al.*, 1975; Sournia & Ricard, 1975, 1976; Sournia, 1976a, b; Ricard, 1976, 1977), and that productivity of water passing over reefs generally increases 2- to 5-fold. Bacterial biomass increases similarly (Sorokin, 1973; Moriarity, 1979). Plankton productivity on and behind coral reefs is a small multiple of productivity in the surrounding ocean waters. Thus, Fig. 1 is suitable for estimating relative plankton primary productivity for coral reefs in different geographic regions.

The highest densities of boring bivalves occur in corals from eastern Pacific locations where waters are enriched by upwelling (Forsbergh, 1963; Smayda, 1966; Wooster & Guillen, 1974; Glynn, 1977; Glynn & Macintyre, 1977). Significantly more western Atlantic corals contained boring bivalves than did Indo-west Pacific corals and a larger proportion of western Atlantic reefs are in areas of high productivity (Fig. 1). Similarly, more Indian Ocean reefs are in regions of high productivity than are western Pacific reefs (Fig. 1).

The significant relationship between abundance of boring bivalves and plankton productivity (Fig. 3) supports the hypothesis that bioerosional damage to coral skeletons by boring organisms is a function of plankton primary productivity. The correlations of non-boring bivalve, ahermatypic coral, serpulid, pyrgomatine and

acorn barnacle abundances with plankton productivity (Fig. 4) serve as independent tests of the hypothesis. These are not boring organisms but, like boring bivalves and sponges, are planktivorous.

The large-scale correlation of borer abundance with productivity suggests that a similar correlation should obtain on a local scale. As water crosses reefs and productivity increases (see above), the abundance of boring sponges and bivalves, with concomitant damage to coral skeletons, should also increase, especially if productivity is raised above 150–200 mg $C \cdot m^{-2} \cdot day^{-1}$.

Fig. 5. *Porites lobata* Dana colony damaged (arrows) by the triggerfish *Pseudobalistes naufragium* (Jordan & Starks, 1895) in order to feed on lithophagine bivalves; note empty *Lithophaga* burrows; photograph by Mark Stouder, Saboga, Pearl Islands, Panama.

Fig. 6. A and B, reef scenes on Belize barrier reef where plankton primary productivity is relatively high (Fig. 1), note presence of gorgonians and sponges, depth ≈15 m; C, windward reef terrace at Enewetak Atoll, Marshall Islands, where plankton productivity is low (Fig. 1); note dominance of corals and lack of other large, sessile organisms; depth ≈ 20 m; D, lagoon pinnacle at Enewetak Atoll, Marshall Islands; depth ≈ 10 m.

Margalef (1968) suggested that hermatypic corals are indicators of infertile waters and that corals are restricted to low latitudes by increased productivity at higher latitudes, rather than temperature. The correlations discussed above suggest the mechanism by which high productivity may restrict coral distribution. They indicate that planktivores will be more abundant on coral reefs where plankton primary productivity is relatively high, and lead to the hypothesis that corals will be progressively less dominant members of the reef community over a gradient of increasing phytoplankton productivity. This increased productivity provides an energy source for a variety of new food chains containing members that (1) burrow into and weaken coral skeletons (Highsmith, 1979), (2) damage corals in order to prey on the planktivorous infauna (Fig. 5; Glynn *et al.*, 1972) and (3) compete with corals for food or space (Fig. 6; Birkeland, 1977). Hence, waters with high phytoplankton productivity should be less favorable locations for hermatypic corals as, for example, found in the Marquesas Islands (Sournia, 1976b) or along the Pacific coast of Panama (Glynn *et al.*, 1972) where reef development is poor.

Conditions favoring high phytoplankton productivity such as elevated nitrogen and phosphate concentrations also favor high productivity by benthic algae (Kinsey & Domm, 1974; Birkeland, 1977). Rapid recruitment and growth of benthic algae reduces the area of hard substratum available for coral larval settlement and may also result in overgrowth of juvenile corals (Adey *et al.*, 1976; Birkeland, 1977; Rogers, 1979). As with phytoplankton, food chains originating with benthic algae may contain members that kill or damage corals. Pomacentrid fish, for example, kill portions of coral colonies within their territories in order to "farm" algae (Kaufman, 1977). In the highly productive area of the Pearl Islands, Panama, pomacentrids are an extremely important source of coral mortality (G. Wellington, P. Glynn, pers. comm.).

Thus, high primary productivity, both planktonic and benthic, appears to affect the distribution of corals by inhibiting coral recruitment and enhancing establishment of food chains containing members that damage or compete with corals. Furthermore, the high phosphate concentrations at locations with high productivity may directly reduce coral growth rates by inhibiting calcium carbonate crystal formation (Kinsey & Davies, 1979). Lower growth rates would impair the ability of corals to compete with benthic algae and filter feeders.

WESTERN ATLANTIC AND INDO-PACIFIC CORALS

While bioerosion is a dynamic process correlated with productivity, there may indeed be a substantial difference between Indo-Pacific and western Atlantic coral species' ability to maintain live tissue over the lower part of their skeletons (Table II). Western Atlantic corals have less of the basal area on their skeleton covered by live tissue, resulting in greater exposure of the base to colonization by boring organisms and weaker basal attachments because the base cannot be enlarged as the

upper part of the head continues to grow. On the average, western Atlantic massive corals have ≈45% less skeletal material in their bases than do western Pacific corals having the same maximum circumference and similar bulk densities. Since this is not simply a characteristic of western Atlantic coral genera (Table III), the causes remain unclear. Some western Atlantic corals exhibit periodic growth (Fig. 7), suggesting there may be unfavorable periods (seasons?) when tissue on the periphery of the colony does not survive. Many colonies of the important reef-building coral *Montastrea annularis*, however, simply maintain live tissue only on the very top of the skeleton, resulting in a columnar growth form (Fig. 4B in Macintyre & Smith, 1974; Fig. 7 in Scatterday, 1974).

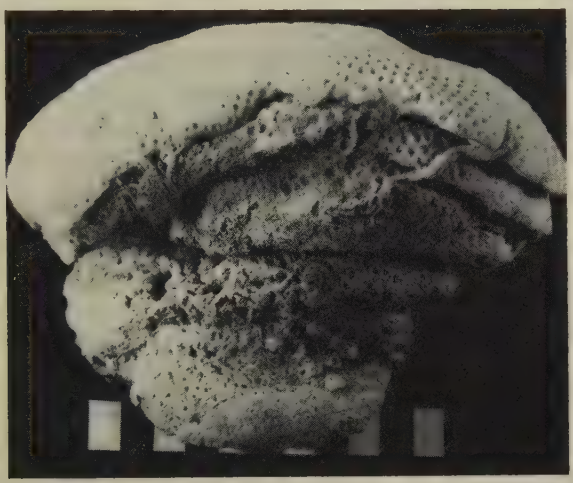


Fig. 7. *Siderastrea siderea* (Ellis & Solander) from −20 m, Morro Grande, Colombia, showing intermittent skeletal deposition: collection by A. Antonius, Jan. 1971; scale bars = 1 cm.

What effect does the generally higher level of bioerosion and weaker basal attachment of massive, western Atlantic corals have on reef development? On the average, more western Atlantic corals should be dislodged by storms than Indo-Pacific corals in storms of equal intensity. At least some Caribbean reefs have, however, grown as fast or faster than Indo-Pacific reefs during the Holocene (Adey, 1978). Apparently, corals and coral reefs recover rather quickly from all but the most intense storms (Stoddart, 1974; Shinn, 1976). Since the drag force per unit area required to detach massive corals is very high for small heads (Highsmith, 1979), western Atlantic corals may become large enough to reproduce and/or survive detachment before they reach a size likely to be broken off by storms. Corals, particularly larger heads and fragments, often survive detachment and continue to grow in new locations (Glynn *et al.*, 1965; Dollar, 1975; Randall & Eldredge, 1977; Highsmith, 1978, 1979; Birkland *et al.*, 1979). At Carrie Bow Cay, Belize, over 40% of

coral colonies and fragments >10 cm in greatest dimension survived detachment during Hurricane Gerta in 1978 (Highsmith *et al.*, in prep.).

The evolutionary reasons for and ecological consequences of Western Atlantic corals retaining live tissue over less of their skeleton than do massive corals elsewhere in the world provide an interesting challenge for future work.

Acknowledgements

Drs. F. M. Bayer, H. Levy, and W. Hartmann provided facilities and access to the coral collections at the Smithsonian Institution, Harvard University and Yale University, respectively. I thank G. Vermeij, E. Zipser, K. Sebens, and V. Tunnicliffe for their generous hospitality during my visits to the museums. M. McBrayer assisted with data analysis. I am indebted to several colleagues for helpful discussion. Drs. A. J. Kohn, R. T. Paine, and an anonymous reviewer suggested improvements in the manuscript. Support was provided by NSF Grant # OCE 76-21271, the Smithsonian Institution, the Mid-Pacific Marine Laboratory, the Graduate School Research Fund of the University of Washington, and D. D. Highsmith.

References

Adey, W. H., 1978. Coral reef morphogenesis: a multidimensional model. *Science, N.Y.*, Vol. 202, pp. 831–837.

Adey, W. H., P. J. Adey, R. Bourke & L. Kaufman, 1976. The Holocene reef systems of eastern Martinique, French West Indies. *Atoll Res. Bull.*, No. 218, pp. 1–40.

Bak, R. P. M., 1976. The growth of coral colonies and the importance of crustose coralline algae and burrowing sponges in relation with carbonate accumulation. *Neth. J. Sea Res.*, Vol. 10, pp. 285–337.

Bertram, G. C. L., 1936. Some aspects of the breakdown of coral at Ghardaqa, Red Sea. *Proc. zool. Soc. Lond.*, Vol. 106, pp. 1011–1026.

Birkeland, C., 1977. The importance of rate of biomass accumulation in early successional stages of benthic communities to the survival of coral recruits. In, *Proc. 3rd int. symp. on coral reefs, Vol. 1*, Rosenstiel School of Marine and Atmospheric Science, Univ. of Miami, U.S.A., pp. 15–21.

Birkeland, C., G. Grimm & R. H. Randall, 1979. Separation of age and colony size as factors influencing the growth rates of corals. Abstract. In, *Use and preservation of coral reef resources*, edited by E. V. Krasnov. XIV Pacific Science Congress, Khabarovsk, U.S.S.R., p. 18.

Connell, J. H., 1973. Population ecology of reef-building corals. In, *Biology and geology of coral reefs. Vol. II. Biology I*, edited by O. A. Jones & R. Endean, Academic Press, New York, pp. 205–245.

Curl, H., Jr., 1960. Primary production measurements in the north coastal waters of South America. *Deep-Sea Res.*, Vol. 7, pp. 183–189.

Dollar, S. J., 1975. Zonation of reef corals off the Kona coast of Hawaii. M.S. thesis, University of Hawaii, Honolulu, 181 pp.

Doty, M. S. & M. Oguri, 1956. The island mass effect. *J. Cons. perm. int. Explor. Mer.*, Vol. 22, pp. 33–37.

Forsbergh, E. D., 1963. Some relationships of meterological, hydrographic, and biological variables in the Gulf of Panama. *Bull. inter-Am. trop Tuna Commn*, Vol. 7, No. 1, pp. 1–109.

Glynn, P. W., 1973. Ecology of a Caribbean coral reef. The *Porites* reef-flat biotope: part II. Plankton community with evidence for depletion. *Mar. Biol.*, Vol. 22, pp. 1–21.

Glynn, P. W., 1977. Coral growth in upwelling and non-upwelling areas off the Pacific coast of Panama. *J. mar. Res.*, Vol. 35, pp. 567–585.

GLYNN, P.W., L. ALMODOVAR & J. GONZALEZ, 1965. Effects of Hurricane Edith on marine life in La Parguera, Puerto Rico. *Caribb. J. Sci.*, Vol. 4, pp. 335–345.

GLYNN, P.W. & I.G. MACINTYRE, 1977. Growth rate and age of coral reefs on the Pacific coast of Panama. In, *Proc. 3rd int. symp. on coral reefs, Vol. 2,* Rosenstiel School of Marine and Atmospheric Science, Univ. of Miami, Miami, U.S.A., pp. 251–259.

GLYNN, P.W., R.H. STEWART & J.E. MCCOSKER, 1972. Pacific coral reefs of Panama: structure, distribution and predators. *Geol. Rdsch.*, Vol. 61, pp. 483–519.

GORDON, D.C., R.O. FOURNIER & G.J. KRASNIC, 1971. Note on the planktonic primary production in Fanning Island lagoon. *Pacif. Sci.*, Vol. 25, pp. 228–233.

HEIN, F. & M. RISK, 1975. Bioerosion of coral heads: inner patch reefs. Florida reef tract. *Bull. mar. Sci.*, Vol. 25, pp. 133–138.

HIGHSMITH, R.C., 1978. Passive colonization and asexual colony multiplication in the massive coral, *Porites lutea*. Abstract. *Am. Zool.*, Vol. 18, p. 662.

HIGHSMITH, R.C., 1979. Corals: The inside story. Ph.D. dissertation, University of Washington, Seattle, 321 pp.

HOLLANDER, M. & D.A. WOLFE, 1973. *Nonparametric statistical methods*. John Wiley and Sons, New York, New York, U.S.A.

JEFFREY, S.W., 1968. Photosynthetic pigments of the phytoplankton of some coral reef waters. *Limnol. Oceanogr.*, Vol. 13, pp. 350–355.

JONES, J.A., 1963. Ecological studies of the southeastern Florida patch reefs. Part I. Diurnal and seasonal changes in the environment. *Bull. mar. Sci.*, Vol. 13, pp. 282–307.

KABANOVA, Y.G., 1968. Primary production of the northern part of the Indian Ocean. *Okeanologiia*, Vol. 8, pp. 270–278.

KAUFMAN, L., 1977. The threespot damselfish: effects on benthic biota of Caribbean coral reefs. In, *Proc. 3rd int. symp. on coral reefs, Vol. 1*, Rosenstiel School of Marine and Atmospheric Science, Univ. of Miami, U.S.A., pp. 559–564.

KIDD, R. & F. SANDER, 1979. Influence of Amazon River discharge on the marine production system off Barbados, West Indies. *J. mar. Res.*, Vol. 37, pp. 567–585.

KINSEY, D.W. & P.J. DAVIES, 1979. Effects of elevated nitrogen and phosphorous on coral reef growth. *Limnol. Oceanogr.*, Vol. 24, pp. 935–940.

KINSEY, D.W. & A. DOMM, 1974. Effects of fertilization on a coral reef environment-primary production studies. In, *Proc. 2nd int. symp. on coral reefs, Vol. 1*, Great Barrier Reef Committee, Brisbane, Australia, pp. 49–66.

KLEEMANN, K., 1977. A new species of *Lithophaga* (Bivalvia) from the Great Barrier Reef, Australia. *Veliger*, Vol. 20, pp. 151–154.

KOBLENTZ-MISHKE, O.J., V.V. VOLKOVINSKY & J.G. KABANOVA, 1970. Plankton primary production of the world ocean. In, *Scientific exploration of the South Pacific*, edited by W.S. Wooster, National Academy of Sciences, Washington, D.C., pp. 183–193.

KREY, J. & B. BABENERD, 1976. *Phytoplankton production: atlas of the international Indian Ocean expedition*. Institut fur Meereskunde an der Universität Kiel. Intergovernmental Oceanographic Comm., UNESCO, Paris, France.

MACGEACHY, J.K. & C. STEARN, 1976. Boring by macro-organisms in the coral *Montastrea annularis* on Barbados reefs. *Int. Revue ges. Hydrobiol. Hydrogr.*, Vol. 61, pp. 715–745.

MACINTYRE, I.G. & S.V. SMITH, 1974. X-radiographic studies of skeletal development in coral colonies. In, *Proc. 2nd int. symp. on coral reefs, Vol. 2*, Great Barrier Reef Committee, Brisbane, Australia, pp. 277–287.

MARGALEF, R., 1968. The pelagic ecosystem of the Caribbean Sea. In, *Symp. on investigations and resources of the Caribbean Sea and adjacent regions*, UNESCO, Paris, France, pp. 483–498.

MARSHALL, N., 1965. Detritus over the reef and its potential contribution to adjacent waters of Eniwetok Atoll. *Ecology*, Vol. 46, pp. 343–344.

MARSHALL, N., A. DURBIN, R. GERBER & G. TELEK, 1975. Observations on particulate and dissolved organic matter in coral reef areas. *Int. Revue ges. Hydrobiol. Hydrogr.*, Vol. 60, pp. 335–345.

MILLIMAN, J.D., 1969. Four southwestern Caribbean Atolls: Courtown Cays, Albuquerque Cays, Roncador Bank and Serrana Bank. *Atoll Res. Bull.*, Vol. 129, pp. 1–26.

MILLIMAN, J.D., 1973. Caribbean coral reefs. In, *Biology and geology of coral reefs. Vol. I. Geology 1*, edited by O. Jones & R. Endean, Academic Press, New York, pp. 1–50.

MORIARITY, D. J. W., 1979. Biomass of suspended bacteria over coral reefs. *Mar. Biol.*, Vol. 53, pp. 193–200.

NIELSEN, C., 1976. Notes on boring bivalves from Phuket, Thailand. *Ophelia*, Vol. 15, pp. 141–148.

QASIM, S. Z. & V. N. SANKARANARAYANAN, 1970. Production of particulate organic matter by the reef on Kavaratti Atoll (Laccadives). *Limnol. Oceanogr.*, Vol. 15, pp. 574–578.

QASIM, S. Z., P. M. A. BHATTATHIRI & C. V. G. REDDY, 1972. Primary production of an atoll in the Laccadives. *Int. Revue. ges. Hydrobiol. Hydrogr.*, Vol. 57, pp. 207–225.

RANDALL, R. H. & L. G. ELDREDGE, 1977. Effects of typhoon Pamela on the coral reefs at Guam. In, *Proc. 3rd int. symp. on coral reefs., Vol. 2.* Rosenstiel School of Marine and Atmospheric Science, Univ. of Miami, Miami, Florida, U.S.A., pp. 525–531.

REISWIG, H. M., 1971. Particle feeding in natural populations of three marine demosponges. *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 141, pp. 568–591.

RICARD, M., 1976. Production primaire planctonique de trois lagons de l'archipel de la Societé, Polynésie Française. *Cah. Pacif.*, Vol. 19, pp. 383–395.

RICARD, M., 1977. Phytoplankton contribution to primary productivity in two coral reef areas of Fiji Islands and French Polynesia. In, *Proc. 3rd int. symp. on coral reefs, Vol. 1.* Rosenstiel School of Marine and Atmospheric Science, Univ. of Miami, Miami, Florida, U.S.A., pp. 343–348.

RICHARDS, F. A., 1960. Some chemical and hydrographic observations along the north coast of South America. I. Cabos Tres Puntas to Curacao, including the Cariaco Trench and the Gulf of Cariaco. *Deep-Sea Res.*, Vol. 7, pp. 163–182.

ROGERS, C. S., 1979. The effect of shading on coral reef structure and function. *J. exp. mar. Biol. Ecol.*, Vol. 41, pp. 269–288.

SANDER, F. & D. M. STEVEN, 1973. Organic productivity of inshore and offshore waters of Barbados: a study of the island mass effect. *Bull. mar. Sci.*, Vol. 23, pp. 771–792.

SCATTERDAY, J. W., 1974. Reefs and associated coral assemblages off Bonaire, Netherlands Antilles, and their bearing on Pleistocene and recent reef models. In. *Proc. 2nd int. symp. on coral reefs, Vol. 2.* Great Barrier Reef Committee, Brisbane, Australia, pp. 85–106.

SHINN, E. A., 1976. Coral reef recovery in Florida and the Persian Gulf. *Environ. Geol.*, Vol. 1, pp. 241–254.

SMAYDA, T. J., 1966. A quantitative analysis of the phytoplankton of the Gulf of Panama. III. General ecological conditions, and the phytoplankton dynamics at 8°45′N, 79°23′W from November 1954 to May 1957. *Bull. inter-Am. trop. Tuna. Commn*, Vol. 11, No. 5, pp. 355–612.

SOROKIN, Y. I., 1973. Microbiological aspects of the productivity of coral reefs. In, *Biology and geology of coral reefs. Vol. II. Biology 1*, edited by O. A. Jones & R. Endean, Academic Press, New York, pp. 17–45.

SOURNIA, A., 1976a. Oxygen metabolism of a fringing reef in French Polynesia. *Helgoländer wiss. Meeresunters.*, Vol. 28, pp. 401–410.

SOURNIA, A., 1976b. Abondance du phytoplancton et absence de récifs coralliens sur les côtes des îles Marquises. *C. r. hebd. Séanc. Acad. Sci., Paris*, Vol. 282, pp. 553–555.

SOURNIA, A. & M. RICARD, 1975. Phytoplankton and primary productivity in Takapoto Atoll, Tuamotu Islands. *Micronesica*, Vol. 11, pp. 159–166.

SOURNIA, A. & M. RICARD, 1976. Phytoplankton and its contribution to primary productivity in two coral reef areas of French Polynesia. *J. exp. mar. Biol. Ecol.*, Vol. 21, pp. 129–140.

STODDART, D. R., 1974. Post-hurricane changes on the British Honduras reefs: resurvey of 1972. In, *Proc. 2nd int. symp. on coral reefs, Vol. 2.* Great Barrier Reef Committee, Brisbane, Australia, pp. 473–483.

WOOSTER, W. S. & O. GUILLEN, 1974. Characteristics of El-Nino in 1972. *J. mar. Res.*, Vol. 32, pp. 387–404.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 197–217
© Elsevier/North-Holland Biomedical Press

ESTIMATION OF ACTIVITIES OF SOME ENZYMES ASSOCIATED WITH ENERGY-YIELDING METABOLISM IN THE POLYCHAETE, *GLYCERA ALBA* (Müller), AND APPLICATION OF THE METHODS TO THE STUDY OF THE EFFECT OF ORGANIC POLLUTION

J. BLACKSTOCK

The Dunstaffnage Marine Research Laboratory, P.O. Box 3, Oban, Argyll, PA34 4AD, Scotland

Abstract: Methods for coenzyme-linked spectrophotometric assays of activities of several enzymes associated with the energy-yielding metabolism of the predatory polychaete, *Glycera alba* (Müller), have been examined with respect to effects of methods of collection, preservation, and extraction of material, and the composition of assay reaction media on enzyme activities estimated on crude extracts of individual worms. Liquid nitrogen and Drikold (solid CO_2) were equally effective for the preservation of specimens prior to the enzyme assays, and phosphate buffer (0.1 M, pH 7.5) was a generally useful extractant. Effects of reaction conditions on phosphofructokinase activities are detailed. This enzyme had an optimum pH of 8.25 and was inhibited by ATP at pH 6.9 but not at the pH optimum.

Activities of phosphofructokinase, pyruvate kinase, malate dehydrogenase, α-glycerophosphate dehydrogenase, lactate dehydrogenase, phosphoenolpyruvate carboxykinase, citrate synthase, and glutamate dehydrogenase have been assayed in crude extracts of *G. alba* from four sampling stations at various distances from the source of discharge of organic effluent from a seaweed factory into Loch Creran in the west of Scotland. Mean phosphofructokinase activity and to a lesser extent, mean pyruvate kinase activity, were lowest in the group of *G. alba* collected from the location most affected by the organic input.

The results are discussed in relation to the reliability of the enzyme assays, the enzyme activity profile of *G. alba* with respect to its ecology, and the development of a biochemical index of effects of organic pollution on this representative of the "pollution-sensitive" macrobenthic invertebrate species found in unpolluted or "moderately" polluted areas of the marine environment.

INTRODUCTION

In recent years biochemical and physiological criteria have been increasingly applied in investigations of the effects of pollutants on marine fauna. Effects of particular pollutants on enzyme systems have been reported (Engel *et al.*, 1972; Moore *et al.*, 1978; Alayse-Danet *et al.*, 1979). Effects of stress, including pollutant-derived stress, on physiological and biochemical processes in marine invertebrates have also been investigated (Cripps & Reish, 1973; Bayne *et al.*, 1976). The effects of pollution caused by high inputs of organic material from industrial installations, e.g. wood pulp mills, into the marine environment have received considerable attention in numerous studies of the associated successions of the benthic invertebrate populations in affected sediments (for review see Pearson & Rosenberg, 1978). Few biochemical investigations have, however, been directed toward assessment of the effects of or-

ganic enrichment on the metabolism of the benthic fauna, although the desirability of such investigations has frequently been indicated (see e.g., Pearson, 1972).

In this laboratory an attempt is being made to relate biochemical changes in selected species of benthic macrofauna to the effects of organic effluents of industrial origin, which are discharged into the Scottish sea-lochs Eil and Creran. In these lochs changes in macrobenthic faunal populations and sediment conditions have been related to inputs of organic effluent from a wood pulp and paper mill (Loch Eil) and a seaweed processing factory (Loch Creran) (Pearson, 1975; Pearson & Stanley, 1979, unpubl.). Investigation of *G. alba*, a predatory polychaete, which may be considered to be sensitive to the effects of organic pollution because it is unable to survive in the most affected areas of Lochs Eil and Creran, has indicated that activities of certain enzymes associated with energy-yielding metabolism, particularly phosphofructokinase, may be related to the effects of the organic effluent (Blackstock, 1978, 1980).

The application of assays of enzyme activity to the assessment of pollution effect presents certain problems which are associated with the techniques for collection of benthic macrofauna, reliability of the subsequent assays, and assessment of the physiological significance of the data. In this paper the assays of enzyme activities in crude extracts of *G. alba* (Müller) are examined critically and the results are discussed with reference to energy yielding metabolism. Further results are also presented in relation to the changes in enzyme activities observed in *G. alba* from various areas within the spatial gradient of the pollution effect in Loch Creran.

Materials and Methods

Preliminary assessment of methods

Estimates of enzyme activities in extracts of biological material may be influenced by numerous factors associated with collection and manipulation of the material, the preparation of the extracts, and the concentrations of various constituents of the assay reaction solutions. Preliminary studies were, therefore, made to assess the effects of these factors on estimates of enzyme activities in *G. alba*. In the preliminary work particular attention has been directed to study of the estimation of phosphofructokinase activity, which may have particular importance in relation to the detection of effects of organic pollution on the metabolism of *G. alba* (Blackstock, 1978, 1980). Details of preliminary experiments are included in the appropriate sections of the text.

Collection and extraction of material

For the preliminary investigations of analytical methodology immature *G. alba* were obtained from the Loch Eil sampling locations detailed by Blackstock (1978)

and from Loch Creran. Material from each Loch was used for separate experiments. Further information relating to the effect of organic pollution from the seaweed factory at Loch Creran on enzyme activities in *G. alba* was obtained by analyses of several individuals from the sampling Stations H, I, J and K (Fig. 1). Conditions at these sampling stations have been discussed (Blackstock, 1980) and at Station H, some 400 m from the effluent outfall conditions in the sediments are influenced by the effluent input, while Stations I, J, and K are considered to be outwith the area of influence of the particulate organic effluent.

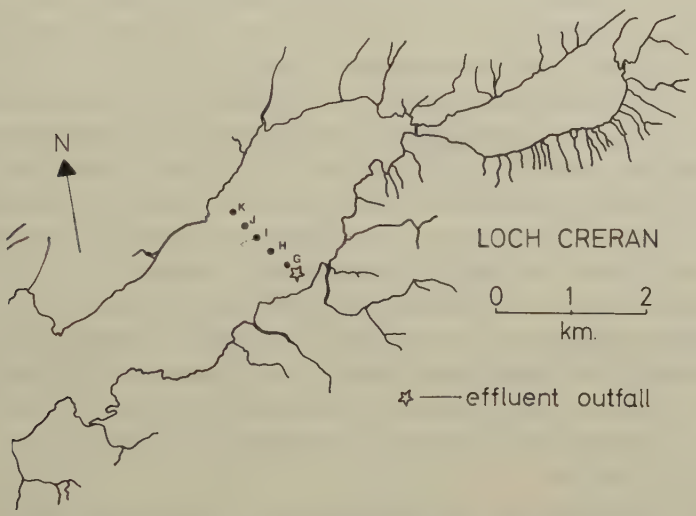


Fig. 1. Outline map of Loch Creran showing the sampling stations.

G. alba were collected by means of a Naturalist's dredge and immediately carefully separated from the sediment by gentle sieving on a 1-mm mesh sieve which retained the worms. For routine estimations of enzyme activity the worms were then rapidly transferred to pre-cooled glass-stoppered test-tubes which were in "dry ice" (Drikold) in an insulated container. Only immature individuals some 2–3 cm in length were selected for analyses.

In the laboratory the quick frozen individuals were transferred to a deep freeze at −25 °C until used. Each animal was then weighed and finely chopped without thawing, into 1- to 2-mm^3 fragments which were then thoroughly mixed. Crude extracts were routinely prepared by homogenizing the individuals in 3 ml 0.1 M phosphate buffer, pH 7.5 for 3 min at 0 °C using a MSE homogenizer (M.S.E. Ltd., Crawley, Sussex, England). The homogenates were centrifuged at 30,000 *g* for 20 min at 0 °C and the supernatant was used for the analyses which were commenced immediately.

Other methods of preservation and extraction were also used in the preliminary investigation of the methods. These are detailed later in the appropriate sections.

ESTIMATIONS OF ENZYME ACTIVITIES

The conditions used for the assays of enzyme activities were as follows. The reactions were monitored at 340 nm (412 nm for citrate synthase) for at least 5 min on a S.P. 800 spectrophotometer (Pye Unicam Ltd., Cambridge) at 25 °C. The enzyme activities are expressed in Units (U) or milli-Units (mU) where 1 Unit represents the conversion of 1 μmol of substrate per min under the test conditions. Variations in test conditions during the preliminary investigation of the methods are given in the appropriate section.

Phosphofructokinase (E.C. No. 2.7.1.11) was assayed by the method described by Ling *et al.* (1966). The assay medium at pH 8.2 contained 53 mM Tris/HCl, 2 mM $MgCl_2$, 2 mM ATP, 50 mM KCl, 1 mM dithiothreitol, 0.16 mM NADH, 100 μg aldolase (0.9 U), 4 μg triose phosphate isomerase (0.19 U), and 4 μg glycerol-3-phosphate dehydrogenase (0.16 U) to which 0.02–0.10 ml of extract was added. The reaction was started by adding fructose-6-phosphate (2 mM). The final volume in the cuvette was 3 ml and control values were obtained by monitoring the decrease in A_{340} for 5 min prior to addition of the fructose-6-phosphate.

Malate dehydrogenase, (NAD) dependent (E.C. No. 1.1.1.37) assays were based on the method described by Ochoa (1955). The assay medium contained 83 mM phosphate buffer, pH 7.4, 0.16 mM NADH, and 0.1 ml of suitably diluted extract. The reaction was started by adding oxaloacetate (1 mM) and the final volume was 3 ml. Control values were obtained by monitoring the decrease in A_{340} prior to addition of oxaloacetate.

Pyruvate kinase (E.C. No. 2.7.1.40) assays were based on the method described by Bucher & Pfleiderer (1955). The assay medium contained 80 mM Tris/HCl pH 7.5, 6.7 mM $MgCl_2$, 67 mM KCl, 2.7 mM phosphoenolpyruvate, 0.16 mM NADH, 250 μg lactate dehydrogenase (140 U), and 0.1 ml extract, suitably diluted as required. The reaction was started by adding ADP (2 mM) and the final volume was 3 ml. Control values were obtained by monitoring the decrease in A_{340} prior to the addition of ADP.

Lactate dehydrogenase (E.C. No. 1.1.1.27) was assayed by the method of Kornberg (1955) in a medium containing 47 mM phosphate buffer, pH 7.5, 0.16 mM NADH and 0.1 ml extract. The reaction was started by adding pyruvate (5 mM). The final volume was 3 ml and control values were obtained by monitoring the decrease in A_{340} prior to the addition of pyruvate.

Phosphoenolpyruvate carboxykinase (E.C. No. 4.1.1.32) assays were based on the method described by Utter & Kurahashi (1954). The assay medium contained 77 mM imidazole buffer, pH 6.5, 0.5 mM $MgCl_2$, 2 mM phosphoenolpyruvate, 2 mM IDP, 0.16 mM NADH, 50 μg malate dehydrogenase (60 U) and 0.1 ml extract. The reaction was started by adding $NaHCO_3$ (50 mM) and the final volume was 3.0 ml. Control values were obtained by monitoring the decrease in A_{340} prior to the addition of $NaHCO_3$.

α-glycerophosphate dehydrogenase (glycerol-3-phosphate dehydrogenase; E.C. No. 1.1.1.8.) assays were based on the method described by Beisenherz *et al.* (1955). The assay medium contained 53 mM Tris/HCl pH 7.5, 0.16 mM NADH and 0.05–0.10 ml extract. The reaction was started by adding dihydroxyacetone phosphate (0.5 mM) and the final volume was 3 ml. Control values were obtained by monitoring the decrease in A_{340} prior to the addition of dihydroxyacetone phosphate.

Glutamate dehydrogenase (E.C. No. 1.4.1.3.) activity assays were done by the method of Schmidt (1974) using reagents obtained in a test-combination (Boehringer Corporation Ltd.). The assay medium contained 50 mM triethanolamine buffer, pH 8.0, 100 mM ammonium acetate, 2.5 mM EDTA, 1.0 mM ADP, 0.2 mM NADH, lactate dehydrogenase (2U), and 0.05–0.10 ml extract. The reaction was started by adding α-oxoglutarate (7 mM) and the final volume was 3.1 ml. Control values were obtained by monitoring the decrease in A_{340} prior to addition of α-oxoglutarate.

Citrate synthase (E.C. No. 4.1.3.7.) assay was based on the spectrophotometric method described by Srere (1969) in which the reaction of 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) with the free thiol group of coenzyme A is monitored at 412 nm. The assay medium contained 90 mM Tris/HCl pH 8.0, 0.05 mM acetyl coenzyme A, 0.2 mM DTNB, and 0.05–0.10 ml extract. The reaction was started by adding oxaloacetate (0.10 mM) and the final volume was 3.0 ml. Control values were obtained by monitoring the increase in A_{412} prior to the addition of oxaloacetate.

Soluble protein in the extracts was assayed by the Lowry method (Lowry *et al.*, 1951).

Results

EFFECTS OF SPECIMEN COLLECTION

Collection of benthic macrofauna incurs short-term metabolic fluctuations as a result of the inevitable disturbance associated with the sampling technique and some assessment of the impact of this disturbance on the observed enzyme activities was attempted. All the *G. alba* from a single trawl were carefully separated from the sediment by gentle sieving and one group of six animals (Group a, Table I) was

Table I

Enzyme activities in extracts of individual *G. alba*: $n = 6$ and the enzyme activities are expressed in mU/mg protein in the extract ± SD; * indicates significant difference between mean enzyme activities in Groups a and b ($P < 0.05$; calculated using Student's *t*-test).

Enzyme	Group a	Group b
Phosphofructokinase	24.2 ± 11.7	22.2 ± 11.1
Pyruvate kinase	277.2 ± 40.0	233.0 ± 74.1
Malate dehydrogenase	804.1 ± 215.8*	1055.8 ± 144.6
α-glycerophosphate dehydrogenase	52.0 ± 12.3	55.3 ± 7.4

frozen in liquid nitrogen. A second group of six animals (Group b, Table I) was transferred to vacuum flasks containing sea water and returned to the laboratory where they were maintained in gently aerated sea water for 24 h before they were rapidly transferred to liquid nitrogen. The enzymes assayed in this experiment were those apparently affected by the effects of organic pollution on *G. alba* from Loch Eil (Blackstock, 1978) and the results shown in Table I indicate that mean phosphofructokinase, pyruvate kinase and α-glycerophosphate dehydrogenase activities were similar in crude extracts of both groups. Mean malate dehydrogenase activity was some 24% lower in Group a than in Group b and application of Student's *t*-test indicated that this difference was significant at $P < 0.05$.

ANALYSES OF FRESH AND FROZEN MATERIAL

Field sampling programmes may require several day's work on location and it is impractical to return all the specimens live to the laboratory for immediate analyses. The specimens must, therefore, be quick-frozen on board ship. Effects of rapid freezing in liquid nitrogen (−196 °C), "dry ice" (Drikold, solid CO_2) (−78 °C), and the slower cooling to −25 °C in a deep freeze have, therefore, been assessed by comparing activities in extracts of specimens cooled by the above methods and in extracts of fresh specimens. Several experiments were done, *G. alba* from a single collection being used for each. After collection the specimens were maintained in sea water, without sediment, for 12 h at 10 °C at which time most of the specimens were rapidly removed and frozen by the appropriate method. Approximately 6 h later the remaining specimens were removed, and immediately extracted. The enzyme

TABLE II

Enzyme activities (mU/mg soluble protein) and soluble protein (mg/g fresh weight of tissue) in extracts of fresh *G. alba* which were preserved by various cooling processes: mean values ±SD are shown with the number of individual worms analysed given in parentheses; * indicates significant differences from mean values obtained with unfrozen specimens ($P < 0.05$, calculated using Student's *t* test).

	Coolant and temperature			
	None	Liquid nitrogen −196 °C	"Dry ice" −78 °C	Deep freeze −25 °C
Phosphofructokinase	61.3 ± 11.9 (5)	*40.6 ± 10.1 (5)	45.2 ± 28.8 (5)	47.7 ± 29.1 (5)
Pyruvate kinase	277.5 ± 78.5 (5)	297.5 ± 31.8 (5)	309.5 ± 107.6 (5)	279.9 ± 144.7 (5)
Malate dehydrogenase	1086.5 ± 234.5 (5)	993.8 ± 162.5 (5)	923.5 ± 208.5 (5)	1029.2 ± 266.9 (5)
α-glycerophosphate dehydrogenase	54.2 ± 7.5 (5)	62.1 ± 6.8 (5)	57.7 ± 8.8 (5)	51.6 ± 7.3 (5)
Lactate dehydrogenase	–	30.5 ± 4.5 (5)	32.2 ± 16.8 (5)	–
Phosphoenolpyruvate carboxykinase	–	1.1 ± 0.6 (4)	1.1 ± 0.1 (4)	–
Citrate synthase	–	7.4 ± 1.9 (4)	6.5 ± 3.1 (5)	–
Glutamate dehydrogenase	–	90.7 ± 21.8 (5)	90.9 ± 41.4 (5)	–
Soluble protein	60.8 ± 10.0 (5)	56.6 ± 9.2 (5)	*43.3 ± 8.5 (5)	51.3 ± 14.1 (5)

analyses of "fresh" and "frozen" specimens were all done at the same time and the results are summarized in Table II. After initial experiments involving assays of phosphofructokinase, pyruvate kinase, malate dehydrogenase, and α-glycerophosphate dehydrogenase activities, further comparisons of the effects of preservation of specimens in liquid nitrogen and in "dry ice" were done. The results of these experiments are also summarized in Table II. Mean phosphofructokinase and malate dehydrogenase activities were highest in the fresh specimens but the differences between treatments were generally small. Only the mean phosphofructokinase activity in specimens cooled in liquid nitrogen and the soluble protein content in the specimens cooled in dry ice were significantly lower ($P < 0.05$) than in the mean values estimated in extracts of fresh specimens. Mean citrate synthase, lactate dehydrogenase, phosphoenolpyruvate carboxykinase, and glutamate dehydrogenase activities were similar in specimens cooled in liquid nitrogen and those cooled in "dry ice".

In general the highest variability within a group was observed in *G. alba* that had been cooled to −25 °C. "Dry ice" or liquid nitrogen were, therefore, considered to be equally suitable for shipboard preservation of *G. alba* for enzyme analyses. The former is more convenient to use on board ship and has been used for routine specimen preservation.

STABILITY OF ENZYME ACTIVITIES IN TISSUES AND EXTRACTS

Although rapid initial cooling of the specimens was essential, subsequent storage at −25 °C or lower temperatures for up to one month had little effect on the enzyme activities and certain enzymes e.g. phosphofructokinase, pyruvate kinase, and glutamate dehydrogenase were apparently stable for much longer periods. After initial cooling to −78 °C and retention at this temperature for at least 24 h the material was, therefore, transferred to a deep freeze at −25 °C to −30 °C where it was stored for limited periods before extraction. As expected, most of the enzymes were labile in the extracts and the degree of lability varied according to the extractant used and the enzyme being extracted. With some enzymes it was possible that lability may have varied in relation to the protein content of the extract. In attempting to assay enzyme activities in extracts on individual worms it was necessary, if several enzyme activities were to be assayed using a single extract, that the protein contents of the extracts in each experiment were comparable. *G. alba* of similar size were, therefore, required and animals 2–3 cm in length, 300 ± 100 mg wet wt yielding extracts containing >1 mg protein/ml were used for estimations of enzyme activities. Under these conditions the activities and half-lives of the more labile enzymes e.g. phosphofructokinase and malate dehydrogenase were independent of the protein content of the extracts. It was desirable to reduce the variation in size between the individuals as much as possible, but any further limitations on selection would have seriously reduced the numbers available from the relatively restricted sampling

areas in Loch Creran to an extent which would have prevented useful inter-station comparisons. The estimations of enzyme activities in the extracts were done in the same order for each batch of analyses with the analyses beginning immediately after the extraction procedure was completed. Phosphofructokinase activity was the most labile and all activity was lost from the extracts in ≈7 h at 4 °C. The decay was linear (see Fig. 2) and activity at zero time was estimated by extrapolation of a plot

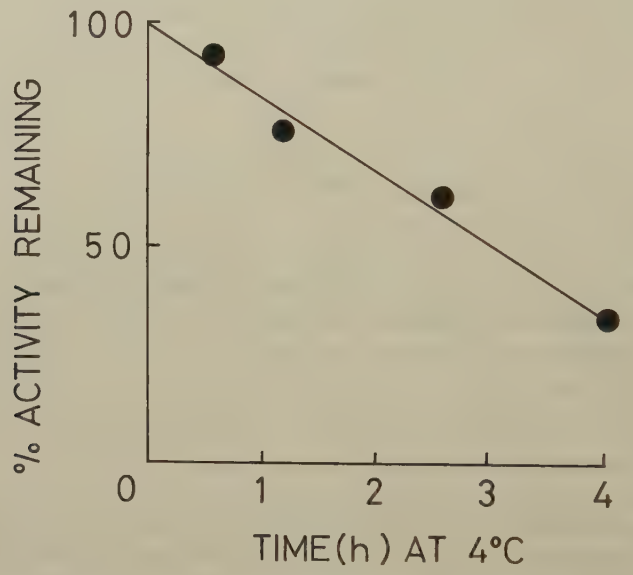


Fig. 2. Loss of phosphofructokinase activity in crude extracts prepared using 0.1 M phosphate buffer, pH 7.5.

of activities measured at a minimum of two different times separated by an interval of 1–2 h. Malate dehydrogenase was the next most labile enzyme and activities were measured immediately after the phosphofructokinase assays were completed. Pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate carboxykinase, and then the apparently completely stable glutamate dehydrogenase activity were assayed in the order stated, the estimations being completed within 60 h. Citrate synthase and α-glycerophosphate dehydrogenase were stable for up to 1 wk in the extracts at −25 °C and consequently a portion of each extract was stored separately at −25 °C for these analyses.

EFFECTS OF VARIOUS EXTRACTANTS

Various extractants were tested to determine optimal extraction conditions but the only two generally suitable ones were 0.1 M phosphate buffer, pH 7.5 and 0.8 M sucrose containing 1 mM dithiothreitol in 1% v/v Triton. An example showing

phosphofructokinase activities recovered with different extractants is shown in Table III. The differences between mean activities obtained in phosphate buffer at pH 7.5 and phosphate buffer + dithiothreitol at pH 8.0 and sucrose-dithiothreitol-Triton were not significant ($P > 0.05$), and any of these three extractants may be

TABLE III

Phosphofructokinase activity (U g fresh tissue) in extracts prepared with different extractants: mean ±SD are shown; $n = 3$.

Extractant	Activity
0.1 M phosphate buffer, pH 7.5	1.40 ± 0.22
0.1 M phosphate buffer, pH 8.0 + 2 mM dithiothreitol	1.71 ± 0.22
0.07 M Tris/HCl, pH 8.2 + 1 mM EDTA + 5 mM $MgCl_2$	0.05 ± 0.04
0.10 M Tris/HCl pH 7.5	0.66 ± 0.07
0.01 M Tris/HCl pH 7.5 + 2 mM EDTA + 2 mM dithiothreitol	0.15 ± 0.05
0.8 M sucrose + 1 mM dithiothreitol in 1% v/v Triton	1.53 ± 0.11
2 g albumin in 100 ml water	1.14 ± 0.15

used for the preparation of crude extracts of *G. alba* for phosphofructokinase assays. Phosphate buffer pH 7.5 was preferred for routine use because efficient extraction and maintenance of the enzymes listed in Table VI was obtained. It was observed, however, that the mean half-life of phosphofructokinase in phosphate buffer pH 7.5 was 3.1 h compared with 5.2 h in the sucrose extractant. The latter extractant has also been found to be superior for certain enzymes in *Capitella capitata* (Blackstock, 1979) and has been successfully used in the preparation of extracts of certain fish tissues (Gould & Nitkowski, 1979). Surprisingly little phosphofructokinase activity was recovered in extracts of *Glycera alba* with 0.07 M Tris/HCl, pH 8.2 containing 1 mM EDTA and 5 mM $MgCl_2$. This extractant was recommended by Opie & Newsholme (1967) and has since been successfully employed for extraction of phosphofructokinase from various invertebrate tissues (Zammit & Newsholme, 1976).

COMPOSITION OF ENZYME ASSAY MEDIA

The final part of this preliminary assessment of methodology involves the selection of the reaction conditions for the assays. The primary objective of the work reported here was the provision of reliable estimates of maximum enzyme activities for comparisons of groups of *G. alba* from the areas being studied. Detailed investigations of the kinetic properties of each enzyme in the crude extracts would have involved a large volume of analytical work, much of which may have yielded little additional information relevant to the objectives of this research. Most of the investigations of reaction conditions have, therefore, involved those enzymes which were considered, after preliminary studies, to be of maximum importance. Phospho-

fructokinase has been investigated in detail and the results are summarized below. Substrate and effector requirements and pH optimum of phosphoenolpyruvate carboxykinase have also been investigated to ensure that the low activities reported (see Table II) were not a consequence of suboptimal reaction conditions. For pyruvate kinase, malate, lactate, α-glycerophosphate dehydrogenases, and citrate synthase only minor modifications of the substrate concentrations have been studied. These tests confirmed that lactate dehydrogenase was not inhibited by excess pyruvate (up to 5 mM), the reactions were of zero order and the reaction rate was proportional to the quantity of extract added. No major fluctuations in activity were observed on reduction of the substrate concentrations given on pp. 200–201 by up to 25% and this was considered to indicate that approximately maximal activities were obtained using the essentially standard spectrophotometric assay procedures detailed on pp. 200–201.

Phosphofructokinase activity in the crude extracts was investigated in the pH range 6.5–8.5 with Tris/HCl (pH 7.0–8.5) and imidazole/HCl (pH 6.5–7.0) buffers at a final concentration of 40 mM in a reaction solution of total volume 1.0 ml containing 0.05–0.10 ml extract prepared in phosphate buffer using pooled *G. alba*. The analyses were done on a LKB 8600 reaction rate analyser (LKB-Produkter, Bromma, Sweden) which was fitted with a cooling unit to facilitate measurements of enzyme activities at 25 °C. The enzyme reactions were monitored for 3 min at 340 nm. Some kinetic properties of phosphofructokinase in crude phosphate buffer extracts of *G. alba* are shown in Fig. 3. The pH optimum of 8.25, inhibition by ATP at pH 6.9, and inhibition by Mg^{2+} at high Mg : ATP concentration ratios at pH 8.25 are typical of phosphofructokinase from various tissues (see Mansour, 1972; Hickman & Wiedemann, 1975). Kinetic studies of phosphofructokinase are being continued with particular emphasis on effects of fructose-6-phosphate concentrations at pH 6.9 in the presence of various effectors. Present investigations show that AMP and cyclic-3, 5-AMP at concentrations of 0.5 2 mM and citrate at 0.5 mM have little effect at pH 8.25 and maximum velocities are, therefore, obtained at pH 8.25 in the presence of 2 mM ATP, 2 mM fructose-6-phosphate, and 2 mM $MgCl_2$. The maximum observed activities at pH 6.9 (at 0.5–1.0 mM ATP) were correlated with maximum observed activities at pH 8.25 for four different specimens which were assayed at pH 6.9 and pH 8.25 in a single batch of analyses. The correlation equation was: activity (mU/ml) at pH 6.9 = 0.42 × activity at pH 8.25 − 1.13 ($r = 0.982$, $P < 0.001$). Activity measured at pH 8.25 under the test conditions showed, therefore, a highly significant correlation with the maximum activity observed at pH 6.9 which represents a closer approximation to physiological conditions under which phosphofructokinase exhibits regulatory properties e.g. inhibition by ATP at concentrations >1 mM.

Phosphoenolpyruvate carboxykinase activities in crude phosphate buffer extracts of *G. alba* were assayed with $MgCl_2$ and $McCl_2$ as the metal activators and the nucleotide requirements were investigated in a preliminary manner with IDP, GDP

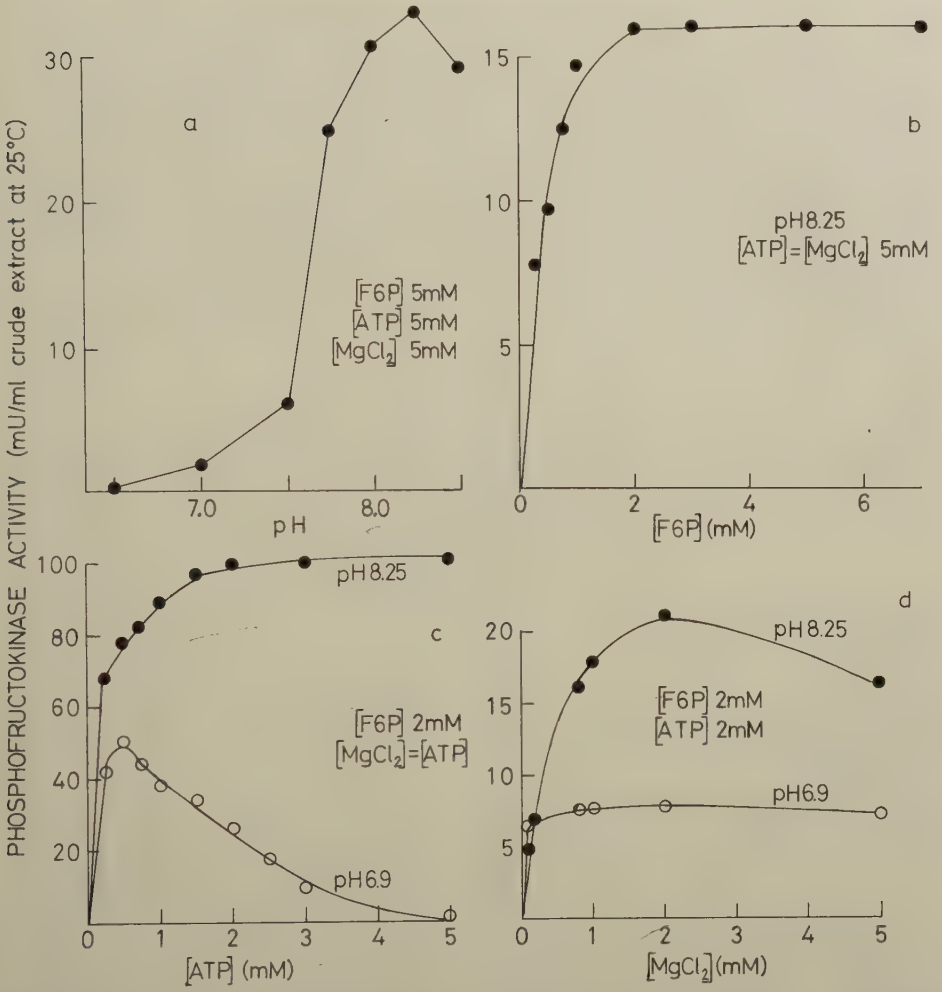


Fig. 3. Phosphofructokinase activity in crude extracts in *G. alba*: a, effect of pH; b, effect of fructose-6-phosphate at the pH optimum; c, effect of ATP at pH 6.9 and 8.25; d, effect of $MgCl_2$ at pH 6.9 and 8.25.

TABLE IV

Phosphoenolpyruvate carboxykinase activities (mU/ml) ± SD in crude extracts of *G. alba*: all assay media contained 2 mM phosphoenolpyruvate, 50 mM $NaHCO_3$ and 0.16 mM NADH; the number of assays are shown in parentheses.

pH	Cation (0.5 mM)	Nucleotide	Activity
6.5	$MgCl_2$	IDP (2 mM)	6.3 ± 2.1 (5)
7.5	$MgCl_2$	IDP (2 mM)	2.1 ± 1.1 (3)
6.5	$MgCl_2$	IDP (1 mM)	2.9 (1)
6.5	$MnCl_2$	IDP (2 mM)	6.3 (2)
6.5	$MgCl_2$	ADP (2 mM)	1.9 (1)
6.5	$MgCl_2$	GDP (2 mM)	25.5 (1)

and ADP. The effect of pH on the reaction was checked using an imidazole buffer, pH 6.5 and Tris/HCl buffer, pH 7.5. The results of these investigations are summarized in Table IV. Activity is higher at pH 6.5, and $MgCl_2$ and $MnCl_2$ are apparently equally effective metal activators. A surprising observation was the approximately four-fold increase in activity when GDP (2 mM) replaced IDP (2 mM) in the reaction solution. The purity of the nucleotides utilized for this comparison was later found to be suspect and consequently the routine assay procedure continues to employ IDP which is the most commonly used nucleotide co-substrate (see de Zwaan, 1977).

TABLE V

Maximal enzyme activities found in *Glycera alba* during the present investigation, in *Nereis virens* and *Aphrodite aculeata* (Zammit & Newsholme, 1976; Alp *et al.*, 1976; Zammit *et al.*, 1978), in *Arenicola marina* (Zebe, 1975), and in *Tubifex tubifex* (Schöttler & Schroff, 1976): the enzyme activities reported for *G. alba* are representative of the maximum mean values from analyses of five individuals from an unpolluted sediment.

	N. virens	*A. aculeata*	*A. marina*	*G. alba*	*G. alba*	*T. tubifex*
	U/g fresh wt				U/g dry wt	
Phosphofructokinase	10.3	8.6	–	3.7	21.8	18.7
Pyruvate kinase	76.0	44.8	8.8	16.9	99.5	55.5
α-glycerophosphate dehydrogenase	0.3	–	0.3	3.5	20.6	–
Lactate dehydrogenase	17.6	0.4	0.4	1.7	10.0	4.0
Phosphoenolpyruvate carboxykinase	0.1	0.1	2.2	0.1	0.6	24.7
Citrate synthase	4.5	0.4	–	0.8	4.7	–
Glutamate dehydrogenase	–	–	0.2	5.1	30.0	–

Table V shows a comparison of "maximal" activities estimated in extracts of *G. alba* and activities estimated in polychaete muscles by Zebe (1975), Zammit & Newsholme (1976), Alp *et al.* (1976), Zammit *et al.* (1978), and in whole *Tubifex tubifex* by Schöttler & Schroff (1976). There is considerable variation between species presumably because of biological differences and some variation in the assay technique. The values obtained with *Glycera alba* are generally within the range observed by the other workers. The highest activities of phosphofructokinase and pyruvate kinase quoted, 10.3 and 76.0 U/g fresh wt, respectively, were found by Zammit *et al.* (1978) who used excised muscle tissue. In *G. alba* all the detectable phosphofructokinase and pyruvate kinase activity was in the muscle tissue of the body wall and proboscis which together accounted for some 75% of the total fresh weight (Blackstock, unpubl.). *G. alba* muscle phosphofructokinase may, therefore, be calculated as ≈ 5 U/g fresh wt and pyruvate kinase as 22.5 U/g fresh wt, values which remain considerably lower than those found in *Nereis virens* and *Aphrodite aculeata*.

ENZYME ACTIVITIES IN *GLYCERA ALBA* FROM LOCH CRERAN

Blackstock (1978, 1980) has indicated that in crude extracts of *G. alba* from various areas of Loch Eil and Loch Creran the activities of phosphofructokinase and pyruvate kinase may reflect effects of conditions in the sediments, largely as a consequence of the organic inputs. Further enzyme activities estimated on crude extracts of individual *G. alba* collected from Stations H, I, J and K in Loch Creran (see Fig. 1) are now given in Table VI. For statistical evaluation logarithmic transformation was applied to the phosphofructokinase, malate dehydrogenase, and citrate synthase activities and square root transformation was applied to the phosphoenolpyruvate carboxykinase activities. Pyruvate kinase, lactate dehydrogenase,

TABLE VI

Mean enzyme activities (mU/mg protein) in extracts of individual *G. alba* collected from Loch Creran in June, 1978: the distance from the point source of particulate organic effluent to each sampling station is also indicated; all values are means ± SD; * indicates significant difference from mean values at Station J ($P < 0.05$, calculated using Student's t test).

Station	H	I	J	K
Distance from effluent outfall (m)	400	650	900	1150
No. of *G. alba*	6	6	5	5
Phosphofructokinase	7.7 ± 5.4*	13.9 ± 6.7	25.2 ± 12.8	17.9 ± 13.2
Pyruvate kinase	218.7 ± 123.3	319.2 ± 99.1	384.0 ± 119.5	387.6 ± 106.2
Malate dehydrogenase	533.2 ± 249.2	900.2 ± 369.8	667.3 ± 358.4	527.3 ± 204.4
α-glycerophosphate dehydrogenase	51.5 ± 18.6	47.6 ± 7.7	54.3 ± 15.5	44.5 ± 12.1
Lactate dehydrogenase	23.4 ± 12.7	31.0 ± 5.9	27.3 ± 10.1	27.9 ± 8.0
Phosphoenolpyruvate carboxykinase	0.1 ± 0.2	0.1 ± 0.3	0.4 ± 0.4	0.6 ± 0.6
Citrate synthase	2.8 ± 5.3	5.4 ± 3.8	4.9 ± 4.7	2.5 ± 5.2
Glutamate dehydrogenase	114.0 ± 33.0	141.6 ± 26.4	139.7 ± 38.2	124.5 ± 20.6

α-glycerophosphate dehydrogenase, and glutamate dehydrogenase activity data did not require transformation. Analyses of variance (Snedecor, 1956) indicated that only phosphofructokinase activities varied significantly ($P < 0.05$) between groups. Application of Student's t test to the phosphofructokinase data indicated that the mean activity in the crude extracts from Station H was significantly lower than the mean activity at Station J. It is also clear that the mean phosphofructokinase activities increase from Stations H to J, and then decline somewhat at Station K where the mean activity is $\approx 70\%$ of the mean activity at Station J, and is similar to the activity observed at Station I. Mean pyruvate kinase activities also increased from Station H to Station J but the difference was of doubtful significance ($0.05 < P < 0.1$).

Discussion

The selective approach used in the preliminary investigations was similar to that used by Zammitt & Newsholme (1976) for the assessment of maximal enzyme activities in muscle tissues dissected from a wide variety of marine invertebrates, and while an attempt has now been made to measure maximal enzyme activities it must be realized that the range of reaction conditions studied was not exhaustive. For example, the pH optima of various marine invertebrate phosphoenolpyruvate carboxykinases are influenced by the metal ion activator (De Zwaan, 1977; Zammit & Newsholme, 1978) and it is possible that a more detailed investigation of *G. alba* phosphoenolpyruvate carboxykinase, possibly including zinc as a metal activator may yield higher activities than the "optimal" conditions selected in this investigation. The low activities of phosphoenolpyruvate carboxykinase in crude extracts of *G. alba* are not considered to be solely a consequence of omission of certain essential components of the reaction mixture because high activities have been found in certain invertebrate tissues (Blackstock, unpubl.) and the phosphoenolpyruvate carboxykinase activities estimated in extracts of *G. alba* are similar to those observed in certain tissues of other polychaete worms (see Table V). Enzymes which are subject to modulation by allosteric affectors e.g. pyruvate kinase and citrate synthase may also exhibit higher observed activities in the presence of effectors which were not included in my preliminary investigations. In the case of phosphofructokinase, however, regulatory properties e.g. inhibition by excess ATP and sigmoidal fructose-6-phosphate saturation curves were not observed at the optimum pH used in the routine assays of activity in the extracts of *G. alba* and it is concluded that maximal activities were measured under the test conditions.

Malate dehydrogenase activities were affected by the sampling procedure (Table I) and phosphofructokinase activities in extracts of frozen *G. alba* were generally 25% lower than in extracts of fresh *G. alba*. These results emphasize the importance of a cautious approach to collection and preservation of material and the subsequent interpretation of the analytical results. In the assessment of the pollution effect all the animals were treated in an identical manner and variations resulting from differences in collection and preservation technique should not have been encountered. Moreover, any variations were not significant in terms of the interpretation of the metabolic significance of the enzyme activities. Phosphofructokinase, pyruvate kinase, and α-glycerophosphate dehydrogenase activities were unaffected by the disturbance associated with the sampling procedure and "dry ice" or liquid nitrogen facilitated adequate preservation of the specimens prior to extraction. The enzyme activities were generally sufficiently non-labile during limited storage of the intact specimens at −25 °C, and in the extracts (see p. 204) for reliable estimates of enzyme activities to be made on the basis of "batch" analyses of *G. alba* collected on each sampling trip. It is probable that optimal extraction and maintenance of each enzyme would require different conditions and that develop-

ment of specialized, and sometimes complex extractants, for each tissue enzyme activity may have slightly improved recoveries of certain of the more labile enzymes. Under these circumstances a separate tissue specimen would be required for each analysis with the disadvantages that several enzymes could not be assayed on a single animal and that rather large quantities of tissue would be required for the range of assays carried out in this investigation. Phosphate buffer (0.1 M, pH 7.5) has been found to be a generally useful extractant even for certain labile enzymes such as phosphofructokinase for which the published optimal pH for maintenance of activity is approximately pH 8 (Ling *et al.*, 1966) (see Table III). The procedure for estimations of enzyme activities in the crude extracts of *G. alba*, represents, therefore, a compromise which is believed to yield reliable estimates of enzyme activity on the extracts with an ease of preparation which permits rapid comparison of animals from the different sampling stations.

ENZYME ACTIVITIES IN *G. ALBA*

The enzyme activities are considered to be maximal within the limitations outlined above and may, therefore, be considered to provide some indication of the capability of *G. alba* for utilization of various metabolic pathways. There are no comparable enzyme activity data for *G. alba* available from the literature but the enzyme activities are generally within the range observed in other polychaetes (see Table V) except that glutamate dehydrogenase and α-glycerophosphate dehydrogenase activities are somewhat higher in *G. alba*. Zammit & Newsholme (1976) and Alp *et al.* (1976) consider that high phosphofructokinase and pyruvate kinase activities, together with low citrate synthase activities are typical of muscles which utilize anaerobic glycolysis for energy production and are capable of short, rapid bursts of contraction. The activities of glycolytic enzymes in entire *G. alba* are derived largely from the muscular body wall and proboscis (Blackstock, unpubl.) and the enzyme activities are considered to be consistent with the predatory mode of life of *G. alba* which is thought to be active in the capture of prey organisms but may remain relatively inactive in its burrow for prolonged periods (Ockelman & Vahl, 1970). The low lactate dehydrogenase activity in extracts of *G. alba* (Table V) is indicative that lactate formation probably contributes little to regeneration of glycolytically formed NADH, and it is possible that the relatively high activities of α-glycerophosphate dehydrogenase may indicate operation of the α-glycerophosphate cycle for NADH regeneration. The high malate dehydrogenase/lactate dehydrogenase ratio is consistent with the operation of the succinate pathway of anaerobic catabolism (Simpson & Awapara, 1966) and it has been shown that high phosphoenolpyruvate carboxykinase activities are not essential for the effective operation of this metabolic pathway (De Zwaan, 1977). A further possibility for maintenance of redox balance is the malate-aspartate shuttle involving aspartate aminotransferase and malate dehydrogenase (Blackstock, 1979). The activities of both these enzymes

are compatible with the opeation of this reaction scheme but aspartate aminotransferase activities are not especially high in *G. alba* compared with other marine invertebrates. Thus, it is clear that *G. alba* possesses adequate cytoplasmic enzyme activities for the regeneration of glycolytically formed NADH, but at present there is no information on the activities of mitochondrial enzymes associated with α-glycerophosphate shuttle, and no attempt has been made to estimate metabolite concentrations. The relative importance of various mechanisms for redox maintenance in *G. alba*, therefore, cannot be evaluated at present.

Glutamate dehydrogenase activity in *G. alba* (Table VI) is apparently high and a possible capability for interconversion of ammonia, α-oxoglutarate, and glutamate is, therefore, indicated. Few polychaete species have been found to contain appreciable glutamate dehydrogenase activity (Jones, 1970; Bishop & Barnes, 1971). Reiss *et al.* (1977) suggest that some of the low glutamate dehydrogenase activities reported in the earlier work may be a consequence of the absence from assay media of ADP, which activates several vertebrate and invertebrate glutamate dehydrogenases.

ENZYME ACTIVITIES IN CRUDE EXTRACTS OF *G. ALBA* FROM LOCH CRERAN

Variations in mean phosphofructokinase and pyruvate kinase activities in crude extracts of *G. alba* from Loch Creran and Loch Eil have been discussed by Blackstock (1978, 1980). A relation between phosphofructokinase activity and the impact of the organic effluent on the sediments in these sea lochs has been observed and the potential usefulness of the assays in assessment of effects of organic pollution on *G. alba*, which is associated with the diverse fauna found in unpolluted and "moderately" polluted marine sediments, has been indicated. It is clear from Table VI that mean phosphofructokinase activity in the crude extracts is the most affected by the impact of the organic effluent at Station H. It is also of interest that in *G. alba* from Station I activities of phosphofructokinase are intermediate between the values obtained from Stations H and J, and an increase in mean activity has, therefore, been observed with increasing distance from the effluent outfall reaching a maximum at Station J, some 900 m from the outfall. Stations I, J and K are considered to be outwith the area of influence of the effluent (T. H. Pearson & S. O. Stanley, unpubl.) and it is possible that the mean phosphofructokinase activity in the extracts of *G. alba* from Station I may reflect some subtle influence which has not been detected by biological and environmental monitoring of the effects of the effluent. The mean phosphofructokinase activity in *G. alba* from Station K, 1150 m from the effluent outfall, is lower than the value found in specimens from Station J. Although the difference is not statistically significant ($P > 0.05$) a necessity for caution in the interpretation of the data in relation to pollution effect is indicated, and further investigations are required for accurate assessment of mean phosphofructo-

kinase activities in relation to spatial gradients of pollution effect and the ecology of *G. alba*. Particular attention is being directed towards further investigation of the possible decrease in activities in *G. alba* from Station K.

Mean activities of most of the enzymes included in this investigation (Table VI) did not change in a manner consistent with responses to the effects of the effluent on *G. alba*. In *G. alba* from Loch Eil low pyruvate kinase and α-glycerophosphate dehydrogenase, and high malate dehydrogenase activities were found in crude extracts of *G. alba* from Station 2 where sediment conditions were considerably affected by the input of effluent from a wood pulp and paper mill (Blackstock, 1978). The mean Eh at 4 cm at Station 2 during the sampling period in 1976–77 was −94 mV and at Loch Creran Station H a mean Eh value of +21 mV was observed during 1976–77 when a regular environmental monitoring programme was in operation. Therefore sediment conditions, exemplified by Eh, at Loch Eil Station 2 were more affected and in fact *G. alba* were visibly stressed on several occasions. It is, therefore, possible that activities of pyruvate kinase, α-glycerophosphate dehydrogenase, and malate dehydrogenase may reflect a response of *G. alba* to more severe pollution effect, while phosphofructokinase activity may represent an initial response to more moderate pollution effects. At present, however, caution must be exercised in the interpretation of the data because of the lack of knowledge relating enzyme activities to temporal fluctuations in pollution effect and ignorance of possible effects of minor, perhaps toxic, components of the different effluents.

METABOLIC SIGNIFICANCE OF PHOSPHOFRUCTOKINASE ACTIVITY

Phosphofructokinase catalyses the conversion of fructose-6-phosphate to fructose diphosphate with the simultaneous conversion of ATP to ADP. The reaction is the first-rate limiting reaction in the glycolytic conversion of glucose-6-phosphate and consequently is of importance in relation to energy yielding catabolism of carbohydrate. The enzyme is sensitive to allosteric modulation by various metabolites including AMP, citrate, ammonia, fructose diphosphate and inorganic phosphate (Mansour, 1972; Newsholme, 1977). The effects of these modulators are generally demonstrable at pH 6.9. At the optimum pH the enzyme from a variety of mammalian tissues is insensitive to allosteric modulation. Mansour (1972) observed that in fresh extracts of mammalian muscle and heart, phosphofructokinase existed in different aggregated forms and he suggested that a pH and ligand directed association–dissociation system could regulate intracellular concentrations of active and inactive forms of the enzyme. The results of the limited investigation of the properties of *G. alba* phosphofructokinase in crude extracts (Fig. 3) have shown pH optimum of 8.25 and inhibition by ATP at pH 6.9; these properties are typical of certain phosphofructokinases of mammalian tissues. Ebberink & Zurburg (1979), however, have observed that ATP inhibition of purified phosphofructokinase from *Mytilus edulis* adductor muscle also occurs at pH values >8. The properties of verte-

brate enzymes clearly cannot be assumed for invertebrate enzymes and any conclusions relevant to regulatory properties of *Glycera alba* phosphofructokinase must be considered to be speculative until a more detailed study of the purified enzyme is done. Investigation of purified regulatory enzymes also presents problems, however, because the purification procedure may affect the regulatory properties and because of this Newsholme & Start (1973) recommend that initial studies of regulatory enzymes be done with crude extracts, because the properties of the enzyme that are relevant to metabolic control are more likely to be preserved. It has been established that maximum activities, in vitro, of certain glycolytic enzymes provide a useful indication of maximum glycolytic flux in muscles from various vertebrates and invertebrates (Crabtree & Newsholme, 1972). Zammit *et al.* (1978) indicate that maximum activities of phosphofructokinase can provide an indication of the maximum flow of metabolites in the glycolytic pathway. The correlation between maximum activities at pH 8.25 and pH 6.9 (see p. 206) suggests that activities under optimal conditions may relate to activities in vivo and it is, therefore, conceivable that phosphofructokinase activities measured in the crude extracts at optimal substrate concentrations and at pH 8.25 may relate to maximum glycolytic flux in *G. alba*.

Bayne *et al.* (1976) suggest that a useful index of pollution should involve a response which may be shown to have a detrimental effect on growth, reproduction or survival. *G. alba* is a predatory polychaete which is thought to rely on intermittent bursts of muscular activity in the capture of its prey, and the construction of a suitable burrow system is a prerequisite for normal behavioural characteristics (Ockelman & Vahl, 1970). An impairment of capability for activity may, therefore, have detrimental consequences for the worms. Blackstock (1978) observed extremely low phosphofructokinase activities (<0.3 mU/mg protein) in crude extracts of *G. alba* from Station 2 in Loch Eil in 1976. At this time conditions in the sediment were considerably affected by the high input of organic effluent from the wood pulp and paper mill. In addition, *G. alba* remained immobile during 24 h of experimentally-induced anoxia and low mean phosphofructokinase activity of 3.6 mU/mg protein was observed at the end of the anoxic period. On the basis of these observations it is speculated that, below a certain threshold value of phosphofructokinase activity there may be some impairment of mobility in *G. alba*, and it is relevant to note that Mansour (1972) indicated that activation of liver fluke phosphofructokinase was accompanied by stimulation of mobility in experimentally maintained individuals. At present, however, it is concluded that the observed fluctuations in phosphofructokinase activity are consistent with a decreased capability for glycolysis in *G. alba* from the areas most affected by inputs of particulate organic effluent into Loch Creran and also Loch Eil (see Blackstock, 1978, 1980). The changes are consistent with detrimental effects of the organic pollution on sediment conditions which affect *G. alba*. Definition of detrimental effects on *G. alba*, indicated by the biochemical changes, must await the outcome of further investigations

of phosphofructokinase activity, measurement of relevant metabolite concentrations and observations of the behaviour of *G. alba* in appropriate experimental situations related to the effects of organic pollution. These investigations are now being commenced in this laboratory and have the primary objective of providing a rapid, reliable, biochemical index of effects of organic pollution on this representative of the "sensitive" macrobenthic invertebrates found in unpolluted or "moderately" polluted areas of the marine environment.

Acknowledgements

I wish to thank Drs. T. H. Pearson, S. O. Stanley and other members of the Organic Degradation Group of the Scottish Marine Biological Association for access to biological and environmental monitoring data relevant to this publication, and for invaluable discussion and comments. I am indebted to Professor T. A. Douglas of the Biochemistry Department, Glasgow University Veterinary School for the provision of facilities for analyses using the reaction rate analyzer. The skilled technical assistance of Mrs. M. MacKay and Miss F. Hamilton is gratefully acknowledged.

References

Alayse-Danet, A. M., J. L. Charlou, M. Jézéquel & J. F. Samain, 1979. Modèle de détection rapide des effets sublétaux des polluants: modification des taux d'amylase et de trypsine d'*Artemia salina* contaminées par le cuivre ou le zinc. *Mar. Biol.*, Vol. 51, pp. 41–46.

Alp, P. R., E. A. Newsholme & V. A. Zammit, 1976. Activities of citrate synthase and NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase in muscle from vertebrates and invertebrates. *Biochem. J.*, Vol. 154, pp. 689–700.

Bayne, B. L., D. R. Livingstone, M. N. Moore & J. Widdows, 1976. A cytochemical and a biological index of stress in *Mytilus edulis* L. *Mar. Pollut. Bull.*, Vol. 7, pp. 221–224.

Beisenherz, G., T. Bücher & K. H. Garbade, 1955. α-glycerophosphate dehydrogenase from rabbit muscle. In, *Methods in enzymology, Vol. 1*, edited by S. P. Colwick & N. O. Kaplan, Academic Press, New York and London, pp. 391–397.

Bishop, S. H. & L. B. Barnes, 1971. Ammonia forming mechanisms: deamination of 5′-adenylic acid (AMP) by some polychaete annelids. *Comp. Biochem. Physiol.*, Vol. 40B, pp. 407–422.

Blackstock, J., 1978. Activities of some enzymes associated with energy yielding metabolism in *Glycera alba* (Müller) from three areas of Loch Eil. In, *Physiology and behaviour of marine organisms, Proc. 12th Europ. mar. Biol. Symp.*, edited by D. S. McLusky & A. J. Berry, Pergamon Press, Oxford and New York, pp. 11–20.

Blackstock, J., 1979. Activities of some enzymes associated with energy yielding metabolism in three ecologically dissimilar species of marine benthic polychaete worms. In, *Abstracts of the 1st ESPCB conference: animals and environmental fitness*, edited by R. Gilles, Pergamon Press, Oxford, pp. 71–72.

Blackstock, J., 1980. A biochemical approach to assessment of effects of organic pollution on the metabolism of the non-opportunistic polychaete, *Glycera alba* (Müller). *Helgoländer wiss. Meeresunters.*, Vol. 33, pp. 122–131.

Bücher, T. & G. Pfleiderer, 1955. Pyruvate kinase from muscle. In, *Methods in enzymology, Vol. 1*, edited by S. P. Colowick & N. O. Kaplan, Academic Press, New York and London, pp. 435–440.

Crabtree, B. & E. A. Newsholme, 1972. The activities of phosphorylase, hexokinase, phosphofructo-

kinase, lactate dehydrogenase and glycerol-3-phosphate dehydrogenase in muscles from vertebrates and invertebrates. *Biochem. J.*, Vol. 126, pp. 49–58.

Cripps, R. A. & D. J. Reish, 1973. The effect of environmental stress on the activity of malate dehydrogenase and lactate dehydrogenase in *Neanthes arenaceodentata*. *Comp. Biochem. Physiol.*, Vol. 46B, pp. 123–133.

De Zwaan, A., 1977. Anaerobic energy metabolism in bivalve molluscs. *Oceanogr. Mar. Biol. Ann. Rev.*, Vol. 15, pp. 103–187.

Ebberink, R. H. M. & W. Zurburg, 1979. Flexibility in anaerobic metabolism in *Mytilus edulis* L. II. Regulatory aspects of glycolysis. In, *Abstracts of the 1st ESPCB conference: animals and environmental fitness*, edited by R. Gilles, Pergamon Press, Oxford, pp. 57–58.

Engel, R. H., M. J. Neat & R. E. Hillman, 1972. Sublethal chronic effects of DDT and Lindane on glycolytic and gluconeogenic enzymes of the quahog, *Mercenaria mercenaria*. In, *Marine pollution and sea life*, edited by M. Ruivo, F.A.O., Fishing News (Books) Ltd., London, pp. 257–260.

Gould, E. & M. Nitkowski, 1979. Changing enzyme activities in maturing gonads of the winter flounder, *Pseudopleuronectes americanus*. In, *Cyclic phenomena in marine plants and animals. Proc. 13th Europ. mar. Biol. Symp.*, edited by E. Naylor & R. G. Hartnoll, Pergamon Press, Oxford, pp. 231–238.

Hickman, P. E. & M. J. Weidemann, 1975. The purification and properties of pig spleen phosphofructokinase. *Biochem. J.*, Vol. 151, pp. 327–336.

Jones, M. L., 1970. The presence of certain dehydrogenases among polychaetous annelids as shown by disc electrophoresis. *Comp. Biochem. Physiol.*, Vol. 36, pp. 605–611.

Kornberg, A., 1955. Lactic dehydrogenase of muscle. In, *Methods in enzymology, Vol. 1*, edited by S. P. Colowick & N. O. Kaplan, Academic Press, New York and London, pp. 441–443.

Ling, K. H., V. Paetkau, F. Marcus & H. A. Lardy, 1966. Phosphofructokinase. I. Skeletal muscle. In, *Methods in enzymology, Vol. 9*, edited by W. A. Wood, Academic Press, New York and London, pp. 425–429.

Lowry, O. H., N. J. Rosebrough, A. L. Farr & R. J. Randall, 1951. Protein measurement with the folin phenol reagent. *J. biol. Chem.*, Vol. 193, pp. 265–275.

Mansour, T. E., 1972. Phosphofructokinase. *Curr. Top. cell. Regul.*, Vol. 5, pp. 1–46.

Moore, M. N., D. M. Lowe & P. E. M. Fieth, 1978. Lysosomal responses to experimentally injected anthracene in the digestive cells of *Mytilus edulis*. *Mar. Biol.*, Vol. 48, pp. 297–302.

Newsholme, E. A., 1977. The regulation of intracellular and extracellular fuel supply during sustained exercise. *Ann. N.Y. Acad. Sci.*, Vol. 301, pp. 81–91.

Newsholme, E. A. & C. Start, 1973. *Regulation in metabolism*. John Wiley & Sons, London, 349 pp.

Ochoa, S., 1955. Malic dehydrogenase from pig heart. In, *Methods in enzymology, Vol. 1*, edited by S. P. Colowick & N. O. Kaplan, Academic Press, New York and London, pp. 735–739.

Ockelman, K. W. & O. Vahl, 1970. On the biology of the polychaete *Glycera alba* especially its burrowing and feeding. *Ophelia*, Vol. 8, pp. 275–294.

Opie, L. H. & E. A. Newsholme, 1967. The activities of fructose-1, 6-diphosphatase, phosphofructokinase and phospnoenolpyruvate carboxykinase in white muscle and red muscle. *Biochem. J.*, Vol. 103, pp. 391–399.

Pearson, T. H., 1972. The effect of industrial effluent from pulp and paper mills on the marine benthic environment. *Proc. R. Soc. Ser. B.*, Vol. 180, pp. 469–495.

Pearson, T. H., 1975. The benthic ecology of Loch Linnhe and Loch Eil, a sea-loch system on the west coast of Scotland. IV. Changes in the benthic fauna attributable to organic enrichment. *J. exp. mar. Biol. Ecol.*, Vol. 20, pp. 1–41.

Pearson, T. H. & R. Rosenberg, 1978. Macrobenthic succession in relation to organic enrichment and pollution of the marine environment. *Oceanogr. Mar. Biol. Ann. Rev.*, Vol. 16, pp. 229–311.

Pearson, T. H. & S. O. Stanley, 1979. A comparative measurement of the redox potential of marine sediments as a rapid means of assessing the effect of organic pollution. *Mar. Biol.*, Vol. 53, pp. 371–379.

Reiss, P. M., S. K. Pierce & S. H. Bishop, 1977. Glutamate dehydrogenase from tissues of the ribbed mussel *Modiolus demissus*: ADP activation and possible physiological significance. *J. exp. Zool.*, Vol. 202, pp. 253–258.

Schmidt, E., 1974. Glutamate dehydrogenase, UV assay. In, *Methods of enzymatic analysis, Vol. 2*, edited by H. U. Bergmeyer, Verlag Chemie Weinheim, 2nd English edition, pp. 650–656.

SCHÖTTLER, U. & G. SCHROFF, 1976. Untersuchungen zum anaeroben Glykogenabbau bei *Tubifex tubifex* M. *J. comp. Physiol.*, Vol. 108, pp. 243–254.

SIMPSON, J. W. & J. AWAPARA, 1966. The pathway of glucose degradation in some invertebrates. *Comp. Biochem. Physiol.*, Vol. 18, pp. 537–548.

SNEDECOR, G. W., 1956. *Statistical methods.* The Iowa State College Press, Iowa, 485 pp.

SRERE, P. A., 1969. Citrate synthase. In, *Methods in enzymology, Vol. 13*, edited by J. M. Lowenstein, Academic Press, New York and London, pp. 3–11.

UTTER, M. F. & K. KURAHASHI, 1954. Mechanism of action of oxalacetic carboxykinase. *J. biol. Chem.*, Vol. 207, pp. 821–841.

ZAMMIT, V. A., I. BEIS & E. A. NEWSHOLME, 1978. Maximum activities and effects of fructose bisphosphate on pyruvate kinase from muscles of vertebrates and invertebrates in relation to the control of glycolysis. *Biochem. J.*, Vol. 174, pp. 989–998.

ZAMMIT, V. A. & E. A. NEWSHOLME, 1976. The maximum activities of hexokinase, phosphorylase, phosphofructokinase, glycerol phosphate dehydrogenases, lactate dehydrogenase, octopine dehydrogenase, phosphoenolpyruvate carboxykinase, nucleoside diphosphate kinase, glutamate-oxaloacetate transaminase and arginine kinase in relation to carbohydrate utilisation in muscles from marine invertebrates. *Biochem. J.*, Vol. 160, pp. 447–462.

ZAMMIT, V. A. & E. A. NEWSHOLME, 1978. Properties of pyruvate kinase and phosphoenolpyruvate carboxykinase in relation to the direction and regulation of phosphoenolpyruvate metabolism in muscles of the frog and marine invertebrates. *Biochem. J.*, Vol. 174, pp. 979–987.

ZEBE, E., 1975. In vivo Untersuchungen über den Glucose-Abbau bei *Arenicola marina* (Annelida, Polychaeta). *J. comp. Physiol.*, Vol. 101, pp. 133–145.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 219–229
© Elsevier/North-Holland Biomedical Press

ULTRASTRUCTURE OF THE CHROMATOPHORES OF *PALAEMON AFFINIS* Heilprin (CRUSTACEA: DECAPODA). THE STRUCTURAL BASIS OF PIGMENT MIGRATION

JOHN C. MCNAMARA[1]
Department of Zoology, University of Canterbury, Christchurch, New Zealand

Abstract: The relationships between pigment granules and the prominent smooth endoplasmic reticulum in the chromatophores of the shrimp, *Palaemon affinis* Heilprin, were investigated by transmission electron microscopy. Different types of pigment granules within the chromatophores were found to exhibit a close structural continuity with the cisternal membranes. The membranes of membrane-bound pigment granules were seen to be continuous with those of the ER cisternae, while pigment granules lacking membranes appear to adhere to the external cisternal surfaces. The reticulum, which seems to form a network enmeshing the pigment granules, is proposed to be part of a continuum linking these granules with their translocating force.

INTRODUCTION

The structural basis of pigment granule translocation within chromatophores has been the subject of numerous investigations, both physiological and ultrastructural. Ultrastructural studies have revealed the presence of well-developed microtubular and microfilamentous systems within crustacean (Chassard-Bouchaud & Hubert, 1971; Elofsson & Kauri, 1971; Robison & Charlton, 1973; McNamara & Taylor, in prep.), and vertebrate chromatophores (Bikle *et al.*, 1966; Green, 1968; Wise, 1969; Schliwa & Bereiter-Hahn, 1973a). Physiological studies by Wikswo & Novales (1969, 1972), McGuire & Moellmann (1972), Lambert & Crowe (1973, 1976), Robison & Charlton (1973), Schliwa & Bereiter-Hahn (1973b) and Fingerman *et al.* (1975), based on the microtubule-disrupting effects of colchicine and vinblastine (Borisy & Taylor, 1967a, b; Wilson, 1970) and the microfilament-disrupting effect of cytochalasin B (Wessels *et al.*, 1971), have implicated the observed microtubule and microfilament systems in pigment movement mechanisms. However, data are currently accumulating, suggesting that granule translocating mechanisms are not microtubule based (Green, 1968; Robison & Charlton, 1973; Schliwa & Bereiter-Hahn, 1975; Lambert & Fingerman, 1976, 1978) and that the non-microtubule/microfilament effects of colchicine and cytochalasin B cannot be ignored in the interpretation of inhibitory mechanisms (Bos & Emmelot, 1974; Maetz & Pic, 1977).

[1] Present address: Departamento de Fisiologia, Instituto de Biociências, Caixa Postal 11.461, Universidade de São Paulo, 05421 São Paulo, Brasil.

Regarding an alternative basis for pigment movement mechanisms, the current study will attempt to demonstrate ultrastructurally, a cytoplasmic component of chromatophores that fulfills the requirements of (1) exhibiting a specific structural association with pigment granules, and (2) being capable of conformational transformations. The nature of the endoplasmic reticulum of the chromatophores of the shrimp, *Palaemon affinis* Heilprin, has been investigated in this respect.

Material and Methods

Specimens of the intertidal shrimp, *Palaemon affinis*, were collected from the Kaikoura Peninsula, New Zealand, and maintained according to McNamara (1979). For ultrastructural examination, portions of the branchiostegite, abdominal pleurae, and hind gut containing chromatophores with either fully-dispersed or fully-aggregated pigments were pre-fixed in 0.2 M Na-cacodylate buffered, 2.5% glutaraldehyde-paraformaldehyde solution at 4 °C for 1–1.5 h, post-fixed in 0.2 M Na-cacodylate buffered, 1% osmium tetroxide at 4 °C for 1 h, dehydrated in a cold ethanol series, and embedded in Spurr's (1969) resin. Thin sections were cut with glass knives on an LKB Bromma Ultratome III, stained with uranyl acetate and Reynolds' (1963) lead citrate, and examined in a JEM 100B electron microscope at an accelerating voltage of 80 kV.

To examine the effects of the microtubule depolymerizing agents, colchicine and vinblastine, on pigment movements, portions of epidermis and hind-gut containing chromatophores with maximally dispersed pigments were incubated in saline alone or in saline containing either 1 mM colchicine (Sigma Chemical Co.) or vinblastine sulphate (Sigma Chemical Co.) for 130 min. These portions were then transferred to saline containing a crude aqueous extract of triturated eyestalks removed from light-adapted shrimps (McNamara, 1979) at a concentration of 16 eyestalk equivalents/ml and the chromatophores observed over a 60-min period.

Results

The chromatophores of *P. affinis* contain a system of diverse and highly organized components, predominantly microtubules and smooth endoplasmic reticulum. Since the arrangements of these organelles within the chromatophore will be described elsewhere (McNamara & Taylor, in prep.), only results pertinent to pigment movements will be related here.

In these chromatophores, the endoplasmic reticulum is an organelle system showing remarkable diversity and complexity. In the cell extensions it comprises three structural variants: elongated cisternae arranged parallel to the prominent microtubule bundles; vesicular scalariform elements arranged perpendicularly to the microtubules, and a tri-dimensional network which also ramifies throughout the

cell. These structural elements of the endoplasmic reticulum exhibit continuity with pigment granules to varying degrees. The membranes of the tri-dimensional network are often continuous with the limiting membrane of the paracrystalline pigment granules in epidermal chromatophores (Fig. 1) and with the membrane-bound granules of hind-gut chromatophores (Fig. 2). Cisternae of this network generally

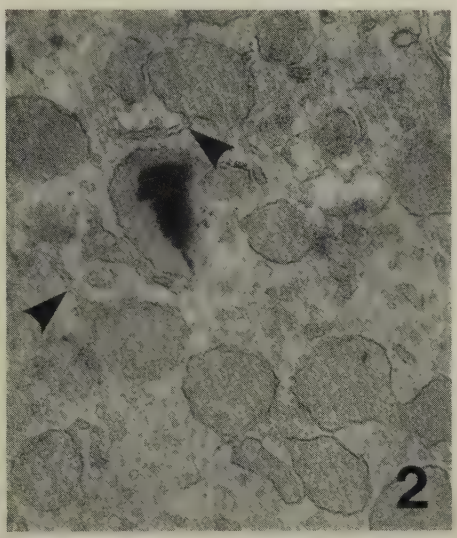


Fig. 1. Continuity (arrows) between limiting-membranes of paracrystals and membranes of tridimensional ER network; ×43,000.

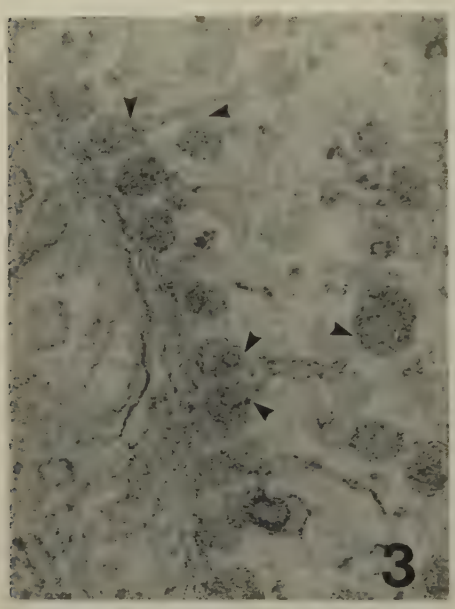
Fig. 2. Continuity (arrows) between pigment granule limiting-membrane and tridimensional ER cisternae; ×43,000.

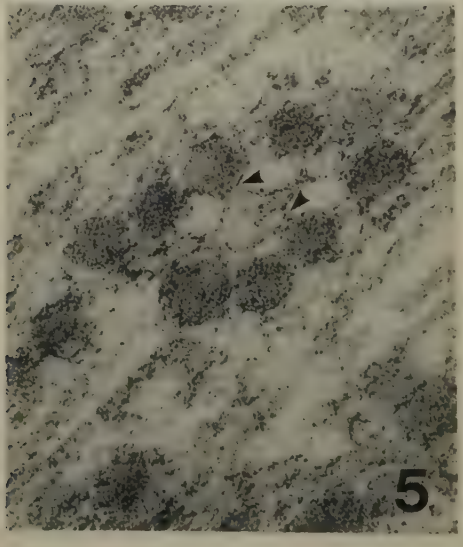
enmesh and also terminate in or on the pigment granules of the amembranous, unstructured type (Figs. 3, 4, 5). These granules were never observed within the intracisternal lumens. Granules of this type also exhibit a close relationship with the small "stalks" and projections derived from the elongated tubular cisternae (Fig. 6). Such cisternae also appear to curl around these granules (Fig. 7), forming a close but unstructured contact. The scalariform elements less frequently show continuity with the pigment granules, but Fig. 8 demonstrates a scalariform element exhibiting well-defined contacts with several granules.

Physiological experiments testing the effects of the microtubule-disrupting agents, colchicine and vinblastine, on the rate of pigment aggregation within chromatophores in response to eyestalk extracts were quantitatively inconclusive. Observations showed, however, that pigment aggregation in response to extracts still occurred in both epidermal and hind-gut chromatophores after incubation in either 1 mM colchicine or vinblastine for 130 min.

Ultrastructurally, colchicine or vinblastine incubations produced several effects.

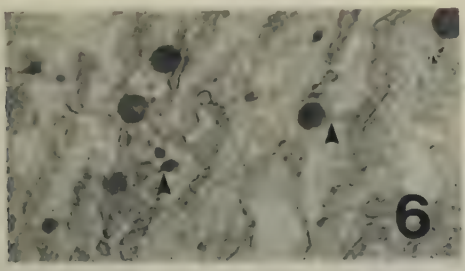
A decreased number of microtubules was observed, their unbroken length (1.06 ± 0.12 μm, mean $\pm$ SE, $n = 50$) being much less than that measured in untreated cells (up to 5 μm). Areas of dense, finely granular material, possibly represen-

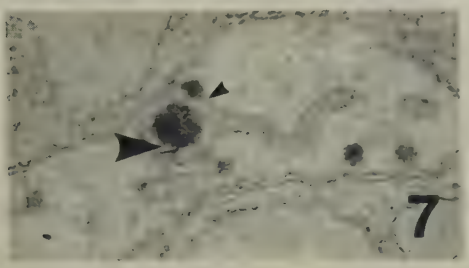




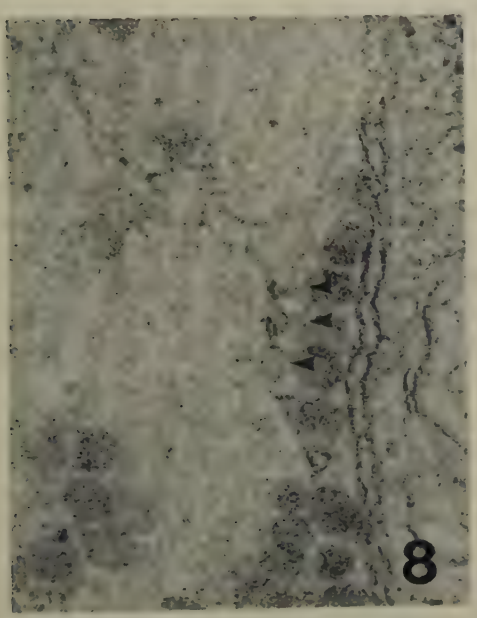
Figs. 3, 4 and 5. Amembranous pigment granules adhering to external cisternal membranes (arrows); Fig. 3 ×75,000; Fig. 4 ×150,000; Fig. 5 ×115,000.

ting aggregates of depolymerized microtubule subunits, were also observable. The most significant effect of colchicine treatment was its effect on the tri-dimensional endoplasmic reticulum network which appears to condense and arrange into an





Figs. 6 and 7. Amembranous pigment-granules attached to stalks (arrows) on elongate ER cisternal system: note cisternum curled around granule (large arrow) in Fig. 7; Fig. 6 ×30,000; Fig. 7 ×43,000.



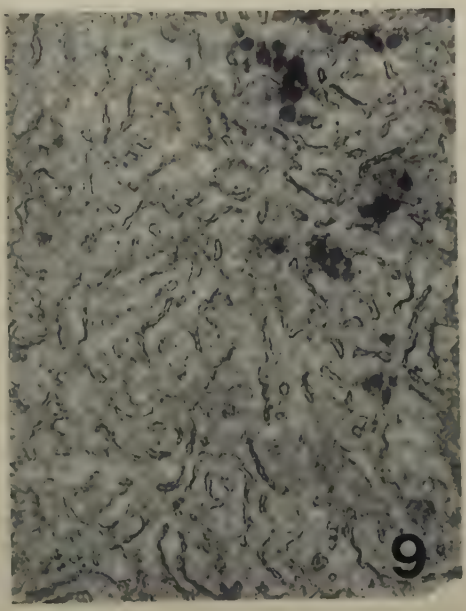


Fig. 8. Several amembranous pigment granules associated with scalariform ER element terminals (arrows); ×90,000.

Fig. 9. Sinuous ER complex appearing after 130 min incubation in colchicine: microtubules are absent from this region; ×40,000.

intricate system of sinuosities (Fig. 9). Microtubules are conspicuously absent from these areas of condensation. In regions containing microtubules, the endoplasmic reticulum does not appear to condense (Fig. 10).

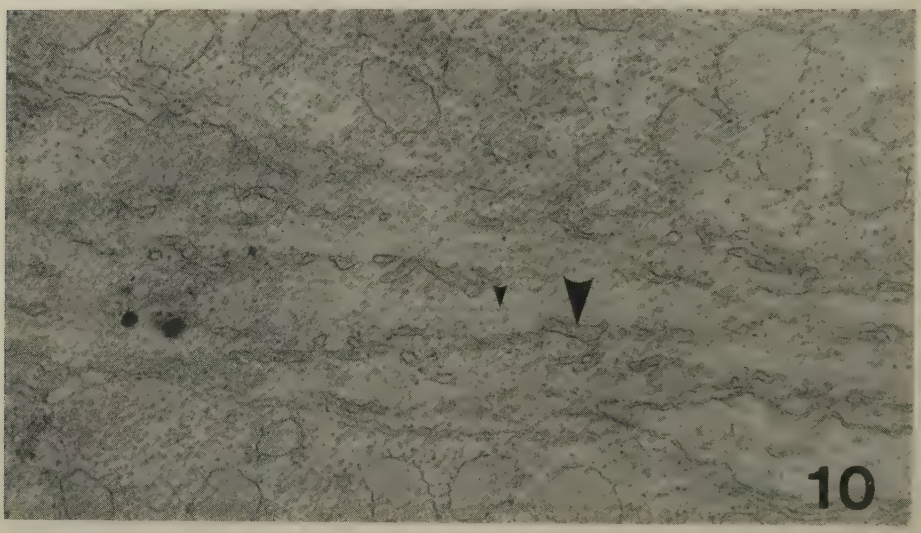


Fig. 10. Elongated cisternae (large arrow) aligned parallel to microtubules (small arrow) in colchicine incubated chromatophore (130 min); ×37,000.

Discussion

In general, investigators have suggested that microtubules provide the motive force for pigment granule translocations within chromatophores, both crustacean (Chassard-Bouchaud & Hubert, 1971) and vertebrate (Schliwa & Bereiter-Hahn, 1973b; Murphy & Tilney, 1974). However, the mechanism of pigment granule movement by microtubules has not been elucidated. Schliwa & Bereiter-Hahn (1973b) have suggested that rapid microtubule turnover (possibly associated with a contractile cellular component) may bring about melanosome movement, while Murphy & Tilney (1974) and Murphy (1975) suggested that microtubular cross-bridges may represent force-generating ATPase molecules which shift pigment granules.

Various ultrastructural factors resulting from several recent studies suggest that microtubules do not directly provide the translocating force for pigment granule movements in *Palaemon affinis* chromatophores. McNamara & Taylor (in prep.) report that microtubules form a central core surrounded by pigment granules within the cell extensions of *P. affinis* epidermal chromatophores containing dispersed pigments. Chassard-Bouchaud & Hubert (1971) reported a similar situation in *Crangon crangon* chromatophores. In such a spatial arrangement, only the pig-

ment granules adjacent to the microtubules would be exposed to the translocating force, those granules shielding other more peripheral granules from any microtubule-localized transportative effect. Since the pigment granules appear to migrate en masse in *Palaemon affinis* chromatophores, it is difficult to envisage bulk movement of granules related to such a spatially isolated translocation center. Given the scarcity and disorganization of microtubules within *P. affinis* hind-gut chromatophores (McNamara, in prep.), their virtual absence from *Hemigrapsus oregonensis* and *Uca pugilator* melanophores (Lambert *et al.*, 1975), and the fact that in *Palaemon affinis* chromatophores some cell extensions do not contain microtubules (McNamara, 1976), a microtubule-located transporting mechanism seems unlikely.

Evidence implicating microtubules in pigment movements both in crustacean and vertebrate chromatophores comes mainly from physiological studies using the microtubule-disrupting agents, colchicine or vinblastine (Wikswo & Novales, 1969, 1972; Lambert & Crowe, 1973, 1976; Schliwa & Bereiter-Hahn, 1973b; Murphy & Tilney, 1974; Castrucci, 1975; Fingerman *et al.*, 1975). In these studies authors have correlated the known microtubule-disrupting effect of colchicine treatment with the concomitant observation of pigment movement inhibition. However, regarding pigment movements in crustacean chromatophores at least, certain anomalies in chromatophore responses present difficulties to the designation of a translocating role for microtubules. Robison & Charlton's (1973) study showed that 1 mM colchicine or vinblastine incubation did not affect the rate of pigment aggregation in *Palaemonetes vulgaris* ovarian chromatophores and that microtubules were not present in the treated cells on electron microscopic examination. The similar results recorded for *Palaemon affinis* chromatophores in the present study also indicate that although most microtubules are disrupted by colchicine treatment, the pigment aggregation mechanism remains intact. Fingerman *et al.* (1975), however, were able to inhibit both pigment dispersion and aggregation in *Palaemonetes vulgaris* chromatophores using 25 mM colchicine. Clearly, since 1 mM colchicine treatment shifts the tubulin-microtubule equilibrium, resulting in a decrease in length and occurrence of microtubules, pigment aggregation, at least, is not dependent on these structures. Fingerman *et al.*'s (1975) concentration-dependent, colchicine-induced inhibition of pigment movements then, may be interpreted as inhibition at a site other than microtubules. Significantly, Lambert & Fingerman (1976) have shown that pigment aggregation in *Uca pugilator* melanophores was inhibited by both colchicine and its derivative lumicolchicine, which does not interact with microtubular protein. Considering this non-microtubular-based inhibition and the rapid reversibility of the colchicine effect in Fingerman *et al.*'s (1975) experiments, it is probable that one site of inhibition is the cell membrane. In fact, evidence for the membrane-function inhibitory effects of colchicine is accumulating. Bos & Emmelot (1974) reported colchicine-mediated inhibition of the Na/K-ATPase, adenylate cyclase, and cyclic-AMP phosphodiesterase activity in rat liver plasma membranes, while Maetz & Pic (1977) demonstrated a colchicine-induced inhibition of Na^+ and Cl^- exchange fluxes

across fish gill chloride cell membranes. These membrane effects are significant in view of Fingerman's (1969) proposed mechanism of the activation of pigment movements in *Palaemonetes vulgaris* erythrophores in which red pigment-dispersing hormone is suggested to increase the rates of flux of Ca^{2+} into the chromatophore activating adenylate cyclase which catalyses cyclic 3′,5′-AMP from ATP. Cyclic 3′,5′-AMP then triggers pigment dispersion. Red pigment-concentrating hormone supposedly stimulates a Na^+/K^+ exchange pump, resulting in a high internal K : Na ratio triggering the pigment aggregating mechanism. On the bases of altered ion permeabilities and an adenylate cyclase inhibitory effect, colchicine may well disrupt pigment movements at the level of the cell membrane before the secondary effect of microtubule disruption is realized. Recently, Lambert & Fingerman (1978) have demonstrated that pigment aggregation can be induced in the erythrophores of *Palaemonetes pugio* by the calcium ionophore A23187, after pre-incubation in colchicine. Since pigment aggregation by the ionophore is initiated at a post-membrane phase in the pigment translocating system, these authors suggested that microtubules are unnecessary for pigment aggregation.

Regarding both the non-specific effects of colchicine and the fact that in many systems microtubules resist colchicine treatment although particle transport is inhibited (Fernandez *et al.*, 1970; Karlsson *et al.*, 1971; Flament-Durand & Dustin, 1972; Schliwa & Bereiter-Hahn, 1973b; Miller & Cawthon, 1974), the general suggestion, based on colchicine treatment alone, that a microtubule-located transporting mechanism is responsible for pigment movements becomes unrealistic.

The structural bases for alternative granule translocating mechanisms are few. Lambert & Crowe (1973, 1976), Robison & Charlton (1973), Fingerman *et al.* (1975), Coohill & Fingerman (1976) and Lambert & Fingerman (1978) have demonstrated the cytochalasin B sensitivity of pigment movements in *Palaemonetes vulgaris*, *Palaemonetes pugio*, *Uca pugilator*, and *Hemigrapsus oregonensis* chromatophores. Based on the proposal of Wessels *et al.* (1971) that cytochalasin B sensitivity implies the presence of a contractile microfilament system, these authors have suggested a microfilament-based, granule-moving apparatus. Again, conflicting physiological and ultrastructural data make this correlation difficult. Microfilaments were not present in any of the chromatophores of *P. affinis* examined nor in the melanophores of *Uca pugilator* or *Hemigrapsus oregonensis* (Lambert *et al.*, 1975) although recently Lambert & Fingerman (1978) have ultrastructurally demonstrated an irregular filamentous component of *Uca pugilator* melanophores by omitting the post-osmification stage of fixation. These authors have also shown that cytochalasin B inhibits A23187-induced pigment aggregation in *Palaemonetes pugio* erythrophores, indicating a possible post-membrane–hormone interaction role for microfilaments in pigment movements although ultrastructural evidence for microfilaments in the erythrophores of this shrimp is still lacking. Robison & Charlton (1973), however, have reported a well-developed microfilament system in *Palaemonetes vulgaris* ovarian chromatophores although the structural integrity of those

filaments was not affected by cytochalasin B treatment. It would be of interest to verify the cytochalasin B-A23187 system in erythrophores from which such a well-developed microfilament system has been described. Cytochalasin B-produced inhibition of pigment movements, like colchicine-induced inhibition, may still be the result of the impairment of cell membrane functions. Bos & Emmelot (1974) demonstrated the cytochalasin B-induced inhibition of the Na/K-ATPase of rat liver membranes while Cohn *et al.* (1972) and Estensen & Plageman (1972) reported competitive inhibition by cytochalasin B in the transport of glucose, deoxyglucose, glucosamine, uridine, and thymine across cell membranes. Estensen & Plageman (1972) suggested that cytochalasin B might affect the general biological functions of cell membranes. This evidence implies that cytochalasin B effects, other than microfilament disruption, such as altered ion permeability of the cell membrane and inhibition of membrane-located enzyme systems, might also account for the inhibition of pigment movements.

Ultrastructural evidence from the current study indicates a further complication regarding pigment granule translocating mechanisms – the involvement of the endoplasmic reticulum. In *Palaemon affinis* chromatophores, a complex structural relationship exists between this organelle system and the various pigment granule types. Considering that many granules are enmeshed by, and actually attached to, the external cisternal membranes and that residual granules are rarely seen in the cell extensions following centripetal pigment migration, it appears that the endoplasmic reticulum itself may be involved in the spatial reorganizations occurring during pigment movements. Green (1968) suggested, but was unable to demonstrate, a continuum comprising pigment granules, the endoplasmic reticulum, and microtubules in *Fundulus heteroclitus* melanophores. The current study provides direct evidence for such a structural continuum. Coiling or contraction of the reticulum, possibly initiated by microtubule depolymerization (or microfilament contraction?) may produce pigment aggregation. Subsequent extension of the continuum and pigment dispersion may be related to microtubule repolymerization. The scalariform cross elements of the reticulum could function as mechanical force transducers, coupling the reticulum with centers of microtubule repolymerization. Although it is not yet clear whether the endoplasmic reticulum could provide the motive force for granule translocations or whether its movements are simply passive, this organelle system is clearly capable of considerable conformational rearrangement and its role in pigment movements must be considered.

That the attachment of the pigment granules to the cisternal membranes could represent a biosynthetic relationship is unlikely. Granules of the unstructured subspherical type were never observed within the cisternal lumens, a more probable site for their synthesis. Additionally, Hubert & Chassard-Bouchaud (1976) suggested that the similar carotenoid pigment granules of *Palaemon serratus* chromatophores are formed by the Golgi apparatus and not the endoplasmic reticulum.

Acknowledgements

This work forms part of a thesis presented to the Department of Zoology, University of Canterbury, New Zealand, under the supervision of Dr. H. H. Taylor to whom the author wishes to express his gratitude. The author would also like to thank Mr. T. Day of the Christchurch Clinical School for kindly providing electron microscopic facilities and Professor Dr. R. J. Lavallard of the Institute of Biosciences, University of São Paulo, for critically reading the manuscript.

References

Bikle, D., L. G. Tilney & K. R. Porter, 1966. Microtubules and pigment migration in the melanophores of *Fundulus heteroclitus*. *Protoplasma*, Vol. 61, pp. 322–345.

Borisy, G. G. & E. W. Taylor, 1967a. The mechanism of action of colchicine. Binding of colchicine-^{3}H to cellular protein. *J. Cell Biol.*, Vol. 34, pp. 525–533.

Borisy, G. G. & E. W. Taylor, 1967b. The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and the mitotic apparatus. *J. Cell Biol.*, Vol. 34, pp. 353–548.

Bos, C. J. & P. Emmelot, 1974. Studies on plasma membranes. XXI. Inhibition of liver plasma membrane enzymes by tumor-promoting phorbolester, mitotic inhibitors and cytochalasin B. *Chem.-Biol. Interactions*, Vol. 8, pp. 349–361.

Castrucci, A. M., 1975. Chromatophores of the teleost *Tilapia melanopleura*. II. The effects of chemical mediators, microtubule disrupting drugs and ouabain. *Comp. Biochem. Physiol.*, Vol. 50A, pp. 457–462.

Chassard-Bouchaud, C. & M. Hubert, 1971. Première données relatives à la presence de microtubules cytoplasmiques dans les chromatophores de Crustacés. *C. R. Acad. Sci. (Paris)*, Vol. 272, pp. 1405–1408.

Cohn, R. H., S. D. Banerjee, E. R. Shelton & M. K. Bernfield, 1972. Cytochalasin B: lack of effect on mucopolysaccharide synthesis and selective alterations in precursor uptake. *Proc. natn. Acad. Sci. U.S.A.*, Vol. 69, pp. 2865–2869.

Coohill, T. P. & M. Fingermann, 1976. Comparison of the effects of illumination on the melanophores of intact and eyestalkless fiddler crabs, *Uca pugilator* and inhibition of the primary response by cytochalasin B. *Experientia*, Vol. 32, pp. 569–570.

Elofsson, R. & T. Kauri, 1971. The ultrastructure of the chromatophores of *Crangon* and *Pandalus*. *J. Ultrastruct. Res.*, Vol. 36, pp. 263–270.

Estensen, R. D. & P. G. Plageman, 1972. Cytochalasin B: inhibition of glucose and glucosamine transport. *Proc. natn. Acad. Sci. U.S.A.*, Vol. 69, pp. 1430–1434.

Fernandez, H. L., F. C. Huneeus & P. F. Davison, 1970. Studies on the mechanism of axoplasmic transport in the crayfish cord. *J. Neurobiol.*, Vol. 1, pp. 395–409.

Fingerman, M., 1969. Cellular aspects of the control of physiological colour changes in crustaceans. *Am. Zool.*, Vol. 9, pp. 443–452.

Fingerman, M., S. W. Fingerman & D. T. Lambert, 1975. Colchicine, cytochalasin B and pigment movements in ovarian and integumentary erythrophores of the prawn *Palaemonetes vulgaris*. *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 149, pp. 165–177.

Flament-Durand, J. & P. Dustin, 1972. Studies on the transport of secretory granules in the magnocellular hypothalmic neurons. I. Action of colchicine on axonal flow and neurotubules in the paraventricular nuclei. *Z. Zellforsch.*, Vol. 130, pp. 440–454.

Green, L., 1968. Mechanism of movements of granules in melanocytes of *Fundulus heteroclitus*. *Proc. natn. Acad. Sci. U.S.A.*, Vol. 59, pp. 1179–1186.

Hubert, M. & C. Chassard-Bouchaud, 1976. Étude ultrastructurale des chromatophores de *Palaemon serratus* Pennant (Crustacé Décapode). Effets de l'ablation des pédoncules oculaires. *Archs Zool. exp. gén.*, Vol. 117, pp. 117–138.

KARLSSON, J. O., H. A. HANSSON & J. SJOSTRAND, 1971. Effect of colchicine on axonal transport and morphology of retinal ganglion cells. *Z. Zellforsch.*, Vol. 115, pp. 265–283.

LAMBERT, D. T. & J. H. CROWE, 1973. Colchicine and cytochalasin B: effects on pigment granule translocation in melanophores of *Uca pugilator* (Crustacea: Decapoda). *Comp. Biochem. Physiol.*, Vol. 46A, pp. 11–16.

LAMBERT, D. T. & J. H. CROWE, 1976. Colchicine, cytochalasin B, cyclic AMP and pigment granule translocation in melanophores of *Uca pugilator* and *Hemigrapsus oregonensis* (Crustacea: Decapoda). *Comp. Biochem. Physiol.*, Vol. 54C, pp. 115–121.

LAMBERT, D. T., J. H. CROWE & M. FINGERMAN, 1975. Cellular aspects of pigment granule translocation in melanophores of *Uca pugilator* and *Hemigrapsus oregonensis*. *Am. Zool.*, Vol. 15, p. 790.

LAMBERT, D. T. & M. FINGERMAN, 1976. Evidence for a non-microtubular colchicine effect in pigment granule aggregation in melanophores of the fiddler crab *Uca pugilator*. *Comp. Biochem. Physiol.*, Vol. 53C, pp. 25–28.

LAMBERT, D. T. & M. FINGERMAN, 1978. Colchicine and cytochalasin B: a further characterization of their actions on crustacean chromatophores using the ionophore A23187 and thiol reagents. *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 155, pp. 563–575.

MALTZ, J. & P. PIC, 1977. Microtubules in the chloride cell of the gill and disruptive effects of colchicine on the sea water adapted *Mugil capito*. *J. exp. Zool.*, Vol. 199, pp. 325–338.

MCGUIRE, J. & G. MOELLMANN, 1972. Cytochalasin B: effects on microfilaments and movement of melanin granules within melanocytes. *Science*, Vol. 175, pp. 642–644.

MCNAMARA, J. C., 1976. The structural basis of pigment migration in the chromatophores of the shrimp *Palaemon affinis*. M.Sc. thesis, University of Canterbury, New Zealand, 2 vols, 71 pp.

MCNAMARA, J. C., 1979. Ultrastructure of the chromatophores of *Palaemon affinis* Heilprin (Crustacea, Decapoda). Modifications in the shape of hindgut chromatophores associated with pigment movements. *J. exp. mar. Biol. Ecol.*, Vol. 40, pp. 193–199.

MILLER, W. H. & D. F. CAWTHON, 1974. Pigment movement in *Limulus* photoreceptors. *Invest. Opthalmol.*, Vol. 13, pp. 401–405.

MURPHY, D. B., 1975. The mechanism of microtubule-dependent movement of pigment granules in teleost melanophores. *Ann. N.Y. Acad. Sci.*, Vol. 253, pp. 692–701.

MURPHY, D. B. & L. G. TILNEY, 1974. The role of microtubules in the movement of pigment granules in teleost melanophores. *J. Cell Biol.*, Vol. 61, pp. 757–779.

REYNOLDS, E. S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.*, Vol. 17, pp. 208–212.

ROBISON, W. G., JR. & J. S. CHARLTON, 1973. Microtubules, microfilaments and pigment movement in the chromatophores of *Palaemonetes vulgaris* (Crustacea). *J. exp. Zool.*, Vol. 186, pp. 279–304.

SCHLIWA, M. & J. BEREITER-HAHN, 1973a. Pigment movements in fish melanophores: morphological and physiological studies. II. Cell shape and microtubules. *Z. Zellforsch. mikrosk. Anat.*, Vol. 147, pp. 107–125.

SCHLIWA, M. & J. BEREITER-HAHN, 1973b. Pigment movements in fish melanophores: morphological and physiological studies. III. The effects of colchicine and vinblastine. *Z. Zellforsch. mikrosk. Anat.*, Vol. 147, pp. 127–148.

SCHLIWA, M. & J. BEREITER-HAHN, 1975. Pigment movements in fish melanophores: morphological and physiological studies. V. Evidence for a microtubule-independent contractile system. *Z. Zellforsch. mikrosk. Anat.*, Vol. 158, pp. 61–73.

SPURR, A. R., 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.*, Vol. 26, pp. 31–43.

WESSELS, N. K., B. S. SPOONER, J. F. ASH, M. O. BRADLEY, M. A. LUDUENA, E. L. TAYLOR, J. T. WREN & K. M. YAMADA, 1971. Microfilaments in cellular and developmental processes. *Science*, Vol. 171, pp. 135–143.

WIKSWO, M. A. & R. R. NOVALES, 1969. The effects of colchicine on migration of pigment granules in the melanophores of *Fundulus heteroclitus*. *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 137, pp. 228–237.

WIKSWO, M. A. & R. R. NOVALES, 1972. Effect of colchicine on microtubules in the melanophores of *Fundulus heteroclitus*. *J. Ultrastruct. Res.*, Vol. 41, pp. 189–201.

WILSON, L., 1970. Properties of colchicine binding protein from chick embryo brain. Interactions with Vinca alkaloids and podophyllotoxin. *Biochemistry*, Vol. 9, pp. 4999–5007.

WISE, G. E., 1969. Ultrastructure of amphibian melanophores after light–dark adaptation and hormonal treatment. *J. Ultrastruct. Res.*, Vol. 27, pp. 472–485.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 231–239
© Elsevier/North-Holland Biomedical Press

QUANTITATIVE STUDIES ON BROWN ALGAL PHENOLS. IV. ULTRAVIOLET SPECTROPHOTOMETRY OF EXTRACTED POLYPHENOLS AND IMPLICATIONS FOR MEASURING DISSOLVED ORGANIC MATTER IN SEA WATER[1]

MARK A. RAGAN and J. S. CRAIGIE
Atlantic Regional Laboratory, National Research Council of Canada, 1411 Oxford Street, Halifax, N.S., Canada B3H 3Z1

Abstract: Poor correlation exists between levels of total phenols and of ultraviolet-absorbing materials in extracts of the brown algae *Ascophyllum nodosum* (L.) Le Jol. and *Fucus vesiculosus* L. The uv extinction coefficient of a major exuded polyphenol fraction increases rapidly and markedly in sea-water solution. Consequently, uv spectrophotometry cannot provide a useful measure of either total polyphenols or total organic matter in extracts or exudates from these brown algae. Moreover, considerable errors may be introduced when uv spectrophotometry is used to estimate dissolved organic matter in sea water which receives significant exudation from brown macrophytes.

INTRODUCTION

It is well established that marine brown algae (Phaeophyceae) can release organic substances into the surrounding sea water (Khailov, 1963a, b; Craigie & McLachlan, 1964; Khailov & Burlakova, 1969; Brylinsky, 1977; Ragan & Jensen, 1979). Among these metabolites are phloroglucinol-based polyphenols (Fretheim, 1974; Sattler, 1974; Ragan, 1976; Glombitza, 1977), which may be present in large quantities in brown seaweeds (Ragan & Jensen, 1978) and which are released into the surrounding sea-water medium during irradiation (Ragan & Jensen, 1979) or following stress or injury to the alga (Sieburth & Jensen, 1970). These polyphenols possess a number of interesting biological properties, including antibacterial (Conover & Sieburth, 1964; Sieburth, 1968) and antilarval (Conover & Sieburth, 1966) activities. Depending on concentration and other factors, the polyphenols may promote (Ragan *et al.*, 1980) or inhibit (McLachlan & Craigie, 1964; Fletcher, 1975) the growth of phytoplankton and macroalgae.

Colorimetric methods have been developed for the estimation of total phenols in extracts of certain brown algae (Ragan & Jensen, 1977, 1978). Only in particularly favourable cases (*e.g.* Sieburth & Jensen, 1968; Ragan & Jensen, 1979) are colorimetric methods directly applicable, however, to the estimation of polyphenols in sea water. A number of factors prevent more widespread or routine application of

[1] NRCC No. 18240.

colorimetric methods, including the insensitivity of the methods, non-linearity of certain reference curves, interferences by inorganic ions, pH effects, lack of suitable reference compounds, and (for the vanillin–sulphuric acid method) the use of corrosive reagents (Ragan & Jensen, 1977).

Hence, interest has developed in ultraviolet (uv) spectrophotometry as a simple alternative to colorimetry. Sea water normally contains uv-absorbing substances (Armstrong & Boalch, 1961; Ogura & Hanya, 1966, 1967), especially near beds of brown algae (Khailov, 1963a, b, 1964; Craigie & McLachlan, 1964; Khailov & Burlakova, 1969). Ultraviolet spectrophotometry has been used to estimate both dissolved organic matter (DOM) (see p. 237) and total organic exudate from brown algae (Langlois, 1975). Furthermore, because polyphenols may constitute a major fraction of the dry matter of some brown seaweeds (Ragan & Jensen, 1977, 1978) and are probably by far the major single group of uv-absorbing substances in brown algal exudates (see Sieburth & Jensen, 1969), it might be thought that by judicious choice of wavelength(s), uv spectrophotometry could provide an approximate measure of polyphenol release from brown seaweeds.

Against this background, we now demonstrate: (a) that poor correlation exists between contents of total extractable phenols and total extractable uv-absorbing materials in *Ascophyllum nodosum* (L.) Le Jol. and *Fucus vesiculosus* L.; (b) that the uv extinction coefficient of a major brown algal polyphenol fraction increases rapidly in sea-water solution; (c) that consequently uv spectrophotometry cannot provide a useful measure of either polyphenols or total organic matter in these exudates; and (d) that there may be considerable over-estimation when uv spectrophotometry is used to estimate DOM in sea water which receives significant exudation from brown macrophytes.

Methods and Materials

Data on seasonal variation of uv-absorbing materials, and on the correlation of uv-absorbing materials with total extractable polyphenols, were obtained from the same extracts of Norwegian *Ascophyllum nodosum* and *Fucus vesiculosus* described earlier (Ragan & Jensen, 1978). Colorimetric determination of polyphenols was made with the Folin–Denis, Brentamine Fast Red 2G Salt, and vanillin–sulphuric acid reagents (Ragan & Jensen, 1977). For experiments with purified high molecular-weight polyphenol fractions, collections were made both at Morris Point (Halifax County, N.S., Canada) and at Flak (Trondheimsfjord, Norway). The polymeric polyphenol fraction was extracted from the fresh alga with aqueous ethanol, and further purified by cellulose column chromatography and dialysis, as described by Ragan & Craigie (1976). The major criterion of purity was the absence of all ^{1}H- and ^{13}C-nuclear magnetic spectroscopic resonances except those in the regions δ_H = 6.0–6.5 ppm and δ_C = 92–103, 123–131, and 148–162 ppm (Ragan *et al.*, 1979).

Values of $E^{1\%(w\ v)}_{260\ nm}$ and $E^{1\%(w\ v)}_{268\ nm}$ (absorption, $\log_{10}(I_0/I)$, of a 1% w/v solution at λ = 260 nm or 268 nm) were calculated for a 10-mm optical path length. For experiments with sea water, these values were determined by mixing equal volumes of the polyphenol solution (in distilled water) with double-concentration sea water (prepared by partial evaporation in vacuo of filtered sea water).

Results

TOTAL UV-ABSORBING MATERIAL AND TOTAL POLYPHENOLS

Ragan & Jensen (1978) have described the seasonal variations of extractable vanillin-, Folin–Denis-, and Brentamine-reactive materials in *Ascophyllum nodosum* and *Fucus vesiculosus*. We now report the uv absorptions (260 and 268 nm) of the same extracts, calculated on a dry weight basis (Fig. 1).

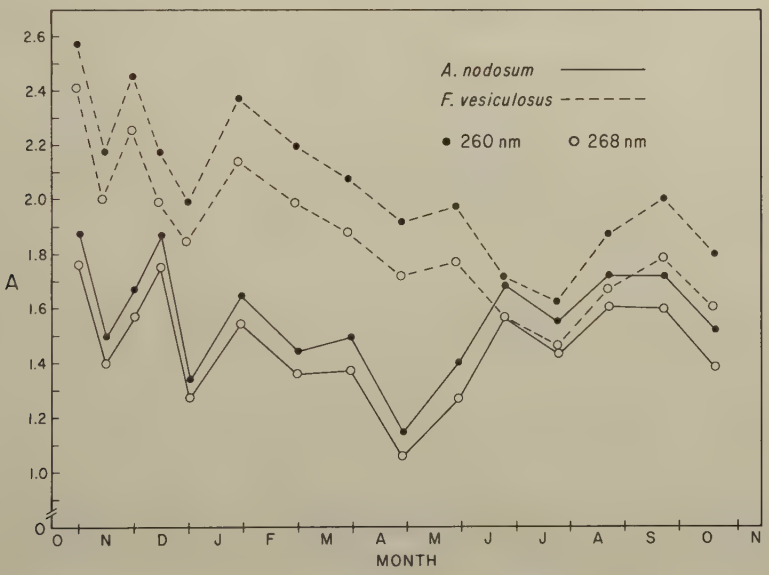


Fig. 1. Seasonal changes in uv absorption of extracts of *F. vesiculosus* and *A. nodosum*: vertical axis, absorption (λ = 260 or 268 nm; optical path length = 10 mm) of a 1-l solution (in distilled water) containing the extract from 1 g (dry wt basis) of algae.

It is evident that a minimum in extractable uv-absorbing materials occurred in April for *Ascophyllum nodosum* and in June–July for *Fucus vesiculosus*. A similar variation was observed in the contents of Brentamine- and Folin–Denis-reactive compounds, where there were seasonal minima in April–May and in May–June for *Ascophyllum nodosum* and *Fucus vesiculosus*, respectively (Ragan & Jensen, 1978). Linear regression of uv absorption on polyphenol concentrations (Table I), how-

ever, yields low values for the correlation coefficient r. Indeed, with the vanillin reaction, the relationship is remarkably random ($|r| < 0.3$ in *F. vesiculosus* extracts). It is not possible, therefore, to calculate accurately the colorimetric values directly

TABLE I

Linear regression, correlation coefficient (r), and estimated standard error of y-intercept (s_a) for relationships between uv absorption and relative contents of polyphenols (as estimated by colorimetry) in extracts from *Ascophyllum nodosum* and *Fucus vesiculosus*: units (for colorimetric data) are mg phloroglucinol equivalents per g algal dry matter; for uv, as in Fig. 1.

uv versus	uv at 260 nm	uv at 268 nm
A. nodosum		
Brentamine	$A_{260} = 0.0103$ (Brent.) + 0.0975 $r = +0.81$ $s_a = \pm 0.2658$	$A_{268} = 0.0097$ (Brent.) + 0.0613 $r = +0.82$ $s_a = \pm 0.1991$
Folin–Denis	$A_{260} = 0.0155$ (F.–D.) + 0.2724 $r = +0.69$ $s_a = \pm 0.2641$	$A_{268} = 0.0146$ (F.–D.) + 0.2336 $r = +0.70$ $s_a = \pm 0.2468$
Vanillin	$A_{260} = 0.1633$ (van.) + 0.2985 $r = +0.45$ $s_a = \pm 0.6116$	$A_{268} = 0.1459$ (van.) + 0.3124 $r = +0.44$ $s_a = \pm 0.5699$
F. vesiculosus		
Brentamine	$A_{260} = 0.0062$ (Brent.) + 0.9490 $r = +0.70$ $s_a = \pm 0.2180$	$A_{268} = 0.0061$ (Brent.) + 0.7744 $r = +0.72$ $s_a = \pm 0.2069$
Folin–Denis	$A_{260} = 0.0133$ (F.–D.) + 0.6561 $r = +0.68$ $s_a = \pm 0.2916$	$A_{268} = 0.0129$ (F.–D.) + 0.5009 $r = +0.68$ $s_a = \pm 0.2801$
Vanillin	$A_{260} = 0.0365$ (van.) + 1.3177 $r = +0.29$ $s_a = \pm 0.4954$	$A_{268} = 0.0315$ (van.) + 1.205 $r = +0.28$ $s_a = \pm 0.4498$

from the uv absorptions. The y-intercept values (Table I) show that the *F. vesiculosus* extracts contain more uv-absorbing nonphenolic material than do the *Ascophyllum nodosum* extracts.

UV ABSORPTION BY BROWN ALGAL POLYPHENOLS IN SEA WATER

Very little is known about the chemical structures or molecular weights of the exuded polyphenols. In investigated brown algae, the polyphenols are largely polymers based on phloroglucinol (1,3,5-trihydroxybenzene) units. Although some monomeric, oligomeric, and sulphated phloroglucinols are present in extracts, much of the total extractable polyphenol fraction is of relatively high molecular weight, including a non-dialysable fraction. The non-dialysable polyphloroglucinol fraction is the major fraction which has been isolated in reasonably pure form (Ragan & Craigie, 1976), and constitutes about 15–25% of the total polyphenol fraction in these two algae. Considerable, albeit circumstantial, evidence (Table II) suggests that polyphloroglucinols of high molecular weight are present in exudates from *A. nodosum* and *Fucus vesiculosus*.

Freshly prepared solutions in distilled water of the non-dialysable polyphloroglucinols give $E^{1\%}_{268}$ values of ≈ 99 (Norwegian *Ascophyllum nodosum*), 119 (Nova

TABLE II

Comparison of properties of non-dialysable "type 2" polymeric polyphenols with those reported for partially purified exudates from *Fucus vesiculosus*.

Property or reaction	Non-dialysable "type 2" polymers from *Fucus vesiculosus*	Non-dialysable "type 2" polymers from *Ascophyllum nodosum*	Exudate from *Fucus vesiculosus* (after Craigie & McLachlan, 1964)
Ultraviolet absorption	λ_{max} = 268 to 269 nm	λ_{max} = 268 nm	λ_{max} = 260 to 280 nm (plateau)
Extinction coefficient (distilled water)	$E^{1\%(w/v)}_{268\ nm,\ 10\ mm}$ = 115 units	$E^{1\%(w/v)}_{268\ nm,\ 10\ mm}$ = 119 units	Not reported
Infrared absorption (cm^{-1})	3400 br, s, 1620 sharp, 1495, 1450, 1370, 1280, 1255, 1145 w, 1025, 995 m, 870 w, 820 s	3400 br, s, 1615 s, 1510, 1495, 1365, 1255, 1205, 1140, 1115, 1030 m, 995 w, 815 m-s, 750 w	3400 br, s, 1615, 1370
Solubility in water	Extremely soluble	Extremely soluble	"Easily soluble"
Solubility in organic solvents	Somewhat soluble in methanol, insoluble in other organic solvents	Somewhat soluble in methanol, insoluble in other organic solvents	Soluble in methanol, insoluble in other organic solvents
Elemental analysis	C 45.12% H 5.36% N 0.16%	C 52.38% H 4.32% N 0.30% S 0.64%	C 48.4% H 4.5% N 0.5%
With $AgNO_3$	Reducing	Reducing	Reducing
With $FeCl_3$	Reducing	Reducing	Reducing
With vanillin–HCl	Red colour at room temperature	Red colour at room temperature	Reddish-orange colour at room temperature
With Ehrlich's reagent	Pink colour	Pink colour	Pink colour
Results of KOH hydrolysis	Phloroglucinol liberated	Phloroglucinol liberated	Phloroglucinol liberated
With polyamide	Binds tightly	Binds tightly	Binds tightly

Scotian *A. nodosum*), and 115 units (Nova Scotian *Fucus vesiculosus*). In sea-water solution, the situation becomes more complex, with the $E^{1\%}_{268}$ of the *Ascophyllum nodosum* polymer increasing rapidly to more than 260 units, and eventually reaching at least 350 units (Fig. 2). As the absorption at 268 nm increases, the absorption at 220 nm

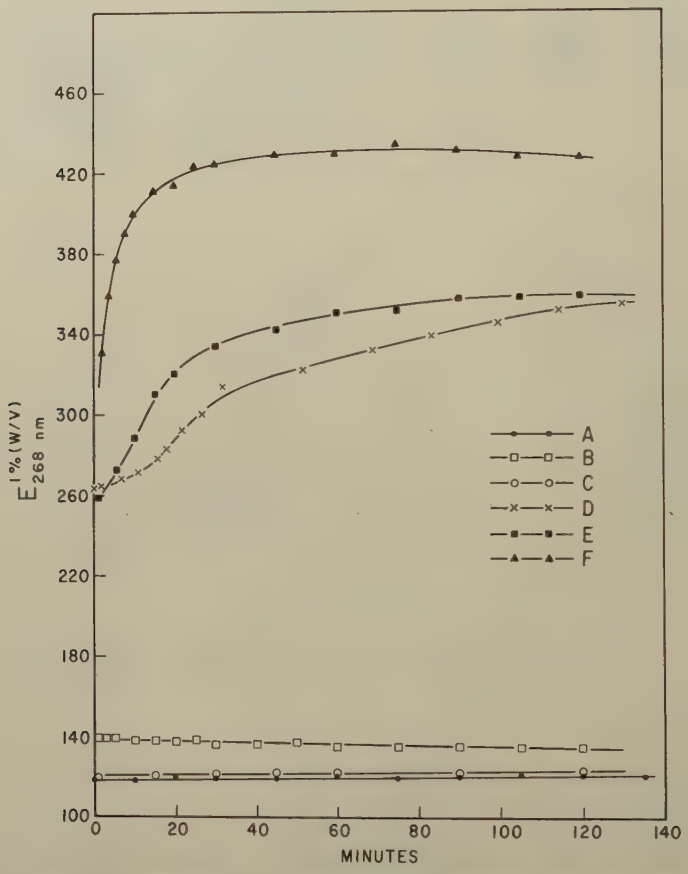


Fig. 2. Temporal variations in the extinction coefficient of the non-dialysable polymer from *A. nodosum* under different conditions: A, glass-distilled water (pH 4.8, 26 °C); B, glass-distilled water adjusted to pH 8.1 with sodium hydroxide (33 °C); C, glass-distilled water (pH 4.8, 45 °C); D, filtered natural sea water (pH 8.1, 33 °C); E, filtered natural sea water (pH 8.1, 45 °C); F, filtered natural sea water adjusted to pH 9.1 with sodium hydroxide (45 °C); values of $E^{1\%(w/v)}_{268\ nm}$ were recorded in a 10 mm quartz cuvette against a glass-distilled water (A–C) or filtered natural sea water (D–F) "blank" in a spectrally matched cuvette using a Unicam SP-8000 spectrophotometer.

decreases, and eventually $E^{1\%}_{268} > E^{1\%}_{220}$. The rates, and to some extent the final magnitudes, of these sea-water-induced absorption changes are increased by elevated temperatures and pH values (Fig. 2). Similar changes have been observed in brown algal exudates (Craigie & McLachlan, 1964; Sieburth & Jensen, 1969).

The pH difference between distilled water and sea water is not the major cause of this rapid sea-water-induced increase in uv absorption; the absorption of the *A. nodosum* polymer in sea-water solution is very much greater than in distilled water adjusted to the same pH (Fig. 2). Solutions of the polymeric polyphenol in Brujewiez's artificial sea water (Sverdrup *et al.*, 1942) also show an increase in uv absorption of the same rate and magnitude as in natural sea water (data not shown).

Discussion

There are severe difficulties involved in the use of uv spectrophotometry to measure the concentration of polyphenols in either extracts or exudates from *A. nodosum* and *Fucus vesiculosus*. Non-phenolic but uv-absorbing compounds are present in extracts, and a poor correlation exists between total polyphenols and uv absorption. In exudates, the uv extinction coefficient of the polyphenols is obviously not constant (Fig. 2).

Polyphenols are thought to comprise ≈15 30% of the total organic exudate from fucoids (Sieburth & Jensen, 1969; Langlois, 1975), and consequently are responsible for much of the uv absorption in these exudates. Wangersky (1978) has noted in this regard that "... an increase in a fraction having a high absorption at that wavelength could lead to a considerable over-estimation of the total release". With brown algal exudates, such increases may arise from increases in the extinction coefficient (Fig. 2), from actual increases in polyphenol release (Ragan & Jensen, 1979), from a decreased extent of precipitation or flocculation reactions (Sieburth & Jensen, 1968, 1969) or from changes in hydrological conditions.

This is of particular interest because spectrophotometric techniques have been used to estimate concentrations of DOM in sea-water samples (Armstrong & Boalch, 1961; Hanya & Ogura, 1964; Khailov & Gorbenko, 1967; Sieburth & Jensen, 1968). For instance, Khailov & Burlakova (1968, 1969) have developed the empirical equation

$$C_{DOM} = 100 \times E_{260}, \quad (1)$$

where C_{DOM} is expressed in mg · l $^{-1}$, and E_{260} is measured against a natural sea-water "blank" in a 5-cm cuvette. Considering the extreme case in which DOM was composed entirely of the *F. vesiculosus* polymeric polyphenol, and assuming that $E^{1\%}_{260} = 350$ units, the relationship between C_{DOM} and E_{260} would actually be

$$C_{DOM} = 5.7 \times E_{260}$$

for a 5-cm path length, and Equation (1) would over-estimate the concentration by a factor of ≈ 100/5.7 = 18. Obviously, such an extreme case is unlikely in nature; nor do these considerations necessarily disprove the validity of Equation (1) or similar equations for estimating DOM in the open ocean. Nevertheless, in areas

where brown algae are locally abundant (as in many inshore environments, fjords, etc.), simple relationships such as Equation (1) may seriously over-estimate DOM concentrations in sea-water samples. The extent of over-estimation is likely to be quite variable, and to depend on a great number of biological, microbiological, chemical, and physical factors (cf. Mattson *et al.*, 1974; Brown, 1977; Wheeler, 1977).

Acknowledgements

Some of this work was completed while M. R. held a National Research Council of Canada postdoctoral fellowship at the Institute of Marine Biochemistry, Trondheim, Norway. We thank Professor A. Jensen for helpful advice during this period.

References

Armstrong, F. A. J. & G. T. Boalch, 1961. The ultra-violet absorption of seawater. *J. mar. biol. Ass. U.K.*, Vol. 41, pp. 591–597.

Brown, M., 1977. Transmission spectroscopy examinations of natural waters. C. Ultraviolet spectral characteristics of the transition from terrestrial humus to marine yellow substance. *Estuar. cstl mar. Sci.*, Vol. 5, pp. 309–317.

Brylinsky, M., 1977. Release of dissolved organic matter by some marine macrophytes. *Mar. Biol.*, Vol. 39, pp. 213–220.

Conover, J. T. & J. McN. Sieburth, 1964. Effect of *Sargassum* distribution on its epibiota and antibacterial activity. *Botanica mar.*, Vol. 6, pp. 147–157.

Conover, J. T. & J. McN. Sieburth, 1966. Effect of tannins excreted from Phaeophyta on planktonic animal survival in tide pools. In, *Proc. Int. Seaweed Symp., Vol. 5*, edited by E. G. Young & J. L. McLachlan. Pergamon Press, Oxford, pp. 99–100.

Craigie, J. S. & J. McLachlan, 1964. Excretion of colored ultraviolet-absorbing substances by marine algae. *Can. J. Bot.*, Vol. 42, pp. 23–33.

Fletcher, R. L., 1975. Heteroantagonism observed in mixed algal cultures. *Nature.* (*Lond.*), Vol. 253, pp. 534–535.

Fretheim, K., 1974. Konstitusjonsstudia av phenoliske forbindelsar frå *Ascophyllum nodosum*. Licentiat thesis, Norges Tekniske Høgskole, Trondheim, 150 pp.

Glombitza, K.-W., 1977. Highly hydroxylated phenols of the Phaeophyceae. In, *Marine natural products chemistry*, edited by D. J. Faulkner & W. H. Fenical, Plenum Press, New York, pp. 191–204.

Hanya, T. & N. Ogura, 1964. Application of ultraviolet spectroscopy to the examination of dissolved organic substances in water. In, *Advances in organic geochemistry*, edited by U. Colombo & G. D. Hobson, MacMillan Co., New York, pp. 447–456.

Khailov, K. M., 1963a. Some unknown organic substances in sea water. *Dokl. Biol. Sci.*, Vol. 147, pp. 1355–1357.

Khailov, K. M., 1963b. Organic exudation of marine macrophytes as a factor in internal inshore communication. *Trudȳ sevastopol'. biol. Sta.*, Vol. 16, pp. 496–505 (in Russian).

Khailov, K. M., 1964. Exudation of organic substances during the life of marine macrophytes, and ecological conditions of the coastal zone. *Trudȳ Murmansk Morsk. Biol. Inst. Akad. SSSR*, Vol. 5, No. 9, pp. 49–56 (in Russian).

Khailov, K. M. & Z. P. Burlakova, 1968. The determination of dissolved organic matter concentration in seawater by direct ultraviolet spectrophotometry. In, *Preprint, 4th Sci. Conf. marine Hydrochem.*, Moscow.

Khailov, K. M. & Z. P. Burlakova, 1969. Release of dissolved organic matter by marine seaweeds and distribution of their total organic production to inshore communities. *Limnol. Oceanogr.*, Vol. 14, pp. 521–527.

KHAILOV, K. M. & YU. A. GORBENKO, 1967. Part played by associations of periphytic microorganisms in ecological metabolism in the sea. *Oceanology*, Vol. 7, pp. 562–568.

LANGLOIS, G. A., 1975. Effect of algal exudates on substratum selection by motile telotrochs of the marine peritrich ciliate *Vorticella marina*. *J. Protozool.*, Vol. 22, pp. 115–123.

MCLACHLAN, J. & J. S. CRAIGIE, 1964. Algal inhibition by yellow ultraviolet-absorbing substances from *Fucus vesiculosus*. *Can. J. Bot.*, Vol. 42, pp. 287–292.

MATTSON, J. S., C. A. SMITH, T. T. JONES & S. M. GERCHAKOV, 1974. Continuous monitoring of dissolved organic matter by UV-visible photometry. *Limnol. Oceanogr.*, Vol. 19, pp. 530–535.

OGURA, N. & T. HANYA, 1966. Nature of ultra-violet absorption of sea water. *Nature* (*Lond.*), Vol. 212, p. 758.

OGURA, N. & T. HANYA, 1967. Ultraviolet absorption of the sea water, in relation to organic and inorganic matters. *Int. J. Oceanol. Limnol.*, Vol. 1, pp. 91–102.

RAGAN, M. A., 1976. Physodes and the phenolic compounds of brown algae. Ph.D. thesis, Dalhousie University, Halifax, 112 pp.

RAGAN, M. A. & J. S. CRAIGIE, 1976. Physodes and the phenolic compounds of brown algae. Isolation and characterization of phloroglucinol polymers from *Fucus vesiculosus* (L.). *Can. J. Biochem.*, Vol. 54, pp. 66–73.

RAGAN, M. A. & A. JENSEN, 1977. Quantitative studies on brown algal phenols. I. Estimation of absolute polyphenol content of *Ascophyllum nodosum* (L.) Le Jol. and *Fucus vesiculosus* (L.). *J. exp. mar. Biol. Ecol.*, Vol. 30, pp. 209–221.

RAGAN, M. A. & A. JENSEN, 1978. Quantitative studies on brown algal phenols. II. Seasonal variation in polyphenol content of *Ascophyllum nodosum* (L.) Le Jol. and *Fucus vesiculosus* (L.). *J. exp. mar. Biol. Ecol.*, Vol. 34, pp. 245–258.

RAGAN, M. A. & A. JENSEN, 1979. Quantitative studies on brown algal phenols. III. Light-mediated exudation of polyphenols from *Ascophyllum nodosum* (L.) Le Jol. *J. exp. mar. Biol. Ecol.*, Vol. 36, pp. 91–101.

RAGAN, M. A., O. SMIDSRØD & B. LARSEN, 1979. Chelation of divalent metal ions by brown algal polyphenols. *Mar. Chem.*, Vol. 7, pp. 265–271.

RAGAN, M. A., C. M. RAGAN & A. JENSEN, 1980. Natural chelators in seawater: detoxification of Zn^{2+} by brown algal polyphenols. *J. exp. mar. Biol. Ecol.*, in press.

SATTLER, E., 1974. Ein neuer Typ gerbstoffartige Polyphenole aus der Braunalge *Halidrys siliquosa*. Doctoral thesis, Rheinischen Friedrich-Wilhelms University of Bonn, 108 pp.

SIEBURTH, J. MCN., 1968. The influence of algal antibiosis on the ecology of marine microorganisms. *Adv. Microbiol. Sea*, Vol. 1, pp. 63–94.

SIEBURTH, J. MCN. & A. JENSEN, 1968. Studies on algal substances in the sea. I. Gelbstoff (humic material) in terrestrial and marine waters. *J. exp. mar. Biol. Ecol.*, Vol. 2, pp. 174–189.

SIEBURTH, J. MCN. & A. JENSEN, 1969. Studies on algal substances in the sea. II. The formation of Gelbstoff (humic material) by exudates of Phaeophyta. *J. exp. mar. Biol. Ecol.*, Vol. 3, pp. 275 289.

SIEBURTH, J. MCN. & A. JENSEN, 1970. Production and transformation of extracellular organic matter from littoral marine algae: a résumé. In, *Symp. on organic matter in natural waters*, edited by D. W. Hood, Univ. Alaska Inst. Mar. Sci. Occas. Publ. 1, pp. 203–223.

SVERDRUP, H. U., M. W. JOHNSON & R. H. FLEMING, 1942. *The oceans: their physics, chemistry, and general biology*. Prentice-Hall, New York, 1087 pp.

WANGERSKY, P. J., 1978. Production of dissolved organic matter. In, *Marine ecology, Vol. 4*, edited by O. Kinne, Wiley-Interscience, New York, pp. 115–220.

WHEELER, J. R., 1977. Dissolved organic carbon: spectral relationships in coastal waters. *Limnol. Oceanol.*, Vol. 22, pp. 573–575.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 241–254
© Elsevier/North-Holland Biomedical Press

RESPIRATORY AND OSMOREGULATORY RESPONSES OF THE HERMIT CRAB, *CLIBANARIUS VITTATUS* (Bosc), TO SALINITY CHANGES

T. D. SABOURIN and W. B. STICKLE

Zoology and Physiology Department, Louisiana State University, Baton Rouge, LA 70803, U.S.A.

Abstract: Intertidal hermit crabs were stepwise acclimated to 10, 20, and 30‰ salinity (*S*) and 21 ± 1 °C. Hemolymph osmolality, sodium, chloride, and magnesium were isosmotic (isoionic) to ambient sea water at 30‰ and hyperosmotic (hyperionic) at 20 and 10‰ *S*, while hemolymph potassium was significantly hyperionic in all acclimation salinities. Total body water did not differ significantly at any acclimation salinity. Oxygen uptake rates were higher in summer- than winter-adapted crabs. No salinity effect on oxygen consumption occurred in winter-adapted individuals. Summer-adapted, 30‰ acclimated crabs had a significantly lower oxygen consumption rate than those acclimated to 10 and 20‰ *S*. Crabs exposed to 30–10 30‰ and 10–30 10‰ semidiurnal (12 h) and diurnal (24.8 h) fluctuating salinity regimes showed variable osmoregulatory and respiratory responses. Hemolymph osmolality followed the osmolality of the fluctuating ambient sea water in all cases, but was regulated hyperosmotically. Hemolymph sodium, chloride, and magnesium concentrations were similar to hemolymph osmolality changes. Sodium levels fluctuated the least. Hemolymph potassium was regulated hyperionically during all fluctuation patterns, but corresponded to sea water potassium only under diurnal conditions. The osmoregulatory ability of *Clibanarius vittatus* (Bosc) resembles that reported for several euryhaline brachyuran species. The time course of normalized oxygen consumption rate changed inversely with salinity under semidiurnal and diurnal 10–30–10‰ *S* fluctuations. Patterns of 30 10–30‰ *S* cycles had no effect on oxygen consumption rate time course changes. The average hourly oxygen consumption rates during both semidiurnal fluctuations were significantly lower than respective control rates, but no statistical difference was observed under diurnal conditions.

INTRODUCTION

The hermit crab, *Clibanarius vittatus* (Bosc), is a common intertidal species along the Louisiana Gulf Coast. In this region it is subjected to large seasonal and diel changes in salinity (Hewatt, 1951; Barrett, 1971; Fotheringham, 1975). Stickle & Howey (1975) have reviewed many of the published data on the common phenomenon of tidal salinity fluctuations in estuaries. Although most tides in the Gulf of Mexico are diurnal, semidiurnal tides also occur (National Ocean Survey, 1977).

Previous studies, mainly with brachyurans, indicate that many decapods accommodate to such salinity alterations with highly efficient physiological adjustments. They are found to maintain a fairly uniform ionic and osmotic internal milieu in the blood which may deviate significantly from the ambient medium (see Lockwood, 1962; Mangum & Towle, 1977). This is thought to be accomplished chiefly by alterations in metabolic processes controlling ion transport (Schoffeniels & Gilles, 1970;

Kinne, 1971), and in the apparent integumental water permeability (Spaargaren, 1975; Smith, 1976).

Anomurans have received little attention with regard to salinity variation (Bryan, 1965; Davenport, 1972a, b, c; Shumway, 1978). The present study was undertaken to assess osmoregulation and rate of oxygen consumption of *C. vittatus* under conditions of steady and fluctuating salinity.

Materials and Methods

Stage C_4-intermolt hermit crabs of dry wt 0.2 to 3.0 g were collected at Grand Isle, Barataria Bay, Louisiana and transported to Louisiana State University in Baton Rouge. Ambient salinity was determined at the time of collection. Hermit crabs were fed clams on a regular schedule while being stepwise acclimated by 2‰ per day increments to a desired salinity by adding deionized water or 40‰ Instant Ocean sea water. The crabs were maintained for at least 7 days at the desired salinity prior to experimentation. All experiments were carried out under continuous light at a temperature of 21 ± 1 °C.

Experiments were carried out in both constant and fluctuating ambient salinity. In constant salinity, measurements were taken on crabs acclimated for at least 7 days and held at either 10, 20, or 30‰ *S*. For the fluctuation experiments, crabs were acclimated to either 10 or 30‰ *S* and cycles of 10–30–10‰ and 30–10–30‰ *S*, respectively, were run over semidiurnal (12 h) or diurnal (24.8 h) periods.

Fluctuating salinity cycles were achieved by changing the salinity from the initial level for either 4 h (semidiurnal) or 10.4 h (diurnal) and holding salinity constant for 2 h to simulate slack water. Salinity was then returned to its initial level over a 4-h or 10.4-h period and again held for a 2-h period. Salinity was increased by pumping 40‰ Instant Ocean sea water into the crab holding tank and decreased by pumping in deionized water at preset flow rates.

For the assessment of osmotic and ionic regulation several crabs were taken from the ambient sea water hourly during semidiurnal cycles and every 2.08 h during diurnal cycles. Crabs were removed from their shells, blotted dry, the wet wt was recorded and hemolymph samples were taken by cardiac puncture with unheparinized microcapillary tubes. Animals were then sexed and dried in an oven to constant weight at 90 °C. Per cent body water was estimated from wet and dry wt.

Hemolymph samples were immediately centrifuged in polyvinyl tubes for 5 min at 10,000 × *g* and an aliquot of the supernatant was diluted for ion analysis. An aliquot of ambient water was also diluted for analysis at each sample interval. The osmolality of hemolymph supernatant and water samples was determined with a Wescor vapor pressure osmometer. Concentrations of sodium and potassium were determined with a Coleman flame photometer, while magnesium ion concentra-

tion was measured using a Perkin Elmer Model 303 atomic absorption spectrophotometer. Chloride ion concentrations were determined with an Aminco chloride titrator.

The effect of constant and fluctuating salinity on rates of oxygen consumption was determined with a flow-through respiration apparatus recently described by Findley *et al.* (1978). Water flow rates of 10–12 ml · min^{-1} were maintained through each experiment. Individual hermit crabs were placed in a respiration chamber and allowed to acclimate for 2 h prior to recording oxygen consumption rates. The crabs moved considerably during the first few minutes, then settled with sensory appendages and mouthparts slightly protruded from the shell. This relaxed position was maintained during all salinity fluctuations as a crab was never observed to retreat into its shell. A control oxygen uptake was determined for each crab at the acclimation salinity. The pen recording for oxygen consumption in the flow-through system allows for continuous monitoring of oxygen removal. It is sensitive enough to detect the presence of short-term breathing rhythms. We have found this phenomenon to occur in various invertebrates; however, no obvious rhythms were observed in *C. vittatus*. During salinity fluctuation the oxygen consumption rates were averaged hourly in semidiurnal and every 2.08 h in diurnal cycles. Additionally, the oxygen consumption rates of several crabs were monitored bihourly during a 24-h period under constant salinity conditions of either 10 or 30‰ *S*. This procedure was undertaken to test for the possible existence of a diurnal respiratory rhythm. Analysis of the results indicated that no rhythm occurred as acclimated crabs in either salinity respired at a uniform level throughout the recording interval.

One-way analysis of variance (ANOVA) was used to determine the significance of variation among osmolality, ion levels, and percent body water at 10, 20, and 30‰ *S*. Orthogonal polynomial comparisons (Steel & Torrie, 1960) were used to test for difference in osmolality, ion levels, and percent body water affected by acclimation to 10, 20, and 30‰ *S*.

To assess the effect of steady-state salinity acclimation on oxygen consumption rate, rate–weight regressions were run for one group of crabs acclimated to 10, 20, and 30‰ *S* during May and June (summer) and another group acclimated to 10 and 30‰ *S* during November and December (winter). If a significant oxygen uptake rate–weight regression existed, individual oxygen consumption rates were corrected for weight as given in Stickle & Sabourin (1979). All salinity effects on oxygen consumption rate were tested by orthogonal polynomial comparisons for a 0.7-g hermit crab. That weight approximated the average weight crab for all respiration experiments.

Osmolality, ion concentrations, per cent body water, and oxygen consumption rates during periods of salinity fluctuation were modelled using maximum R-square improvement regression analysis for the dependent variable (Service, 1972) and are expressed in graphic form as the predicted mean plus or minus the 95% confidence intervals.

TABLE I

Osmotic composition of *Clibanarius vittatus* hemolymph after 7 days acclimation to constant salinity: hemolymph data given as mean ± SE; *n*, sample size; SW, sea water; HL, hemolymph.

		30‰		20‰		10‰		Response significance	
Treatment	Unit	SW	HL	SW	HL	SW	HL	Linear	Quadratic
Osmolality	mOsm	861	923 ± 14 (8)	586	738 ± 8 (10)	306	644 ± 11 (9)	$P < 0.005$	$P < 0.005$
Body water	%	–	63.9 ± 1.3 (10)	–	64.9 ± 1.3 (10)	–	65.4 ± 0.6 (9)	NS	NS
Sodium	mM	349	344 ± 6 (4)	250	309 ± 5 (8)	123	251 ± 4 (4)	$P < 0.005$	NS
Chloride	mM	489	451 ± 25 (4)	295	360 ± 7 (8)	157	219 ± 10 (4)	$P < 0.005$	NS
Magnesium	mM	37.6	34.6 ± 1.1 (4)	24.8	36.1 ± 2.6 (6)	10.1	18.2 ± 1.1 (4)	$P < 0.005$	$P < 0.10$
Potassium	mM	9.1	20.3 ± 1.6 (4)	4.7	13.9 ± 0.9 (8)	3.2	12.9 ± 2.2 (4)	$P < 0.005$	$P < 0.05$

RESULTS

OSMOTIC AND IONIC REGULATION

Hemolymph osmolality, ion concentrations, and per cent body water data for hermit crabs acclimated to 10, 20, and 30‰ *S* are given in Table I. Hemolymph osmolality declined significantly ($P < 0.05$) at reduced ambient salinity, but was 152 milliosmoles · kg $^{-1}$ (mOsm) hyperosmotic at 20‰ *S* and 338 mOsm hyperosmotic at 10‰ *S*. Body water was not significantly different for hermit crabs acclimated to 10, 20, and 30‰ *S*. Steady-state concentrations of Na^+, Cl^-, and Mg^{2+} were essentially isoionic to the medium at 30‰ *S* while K^+ was regulated hyperionically. All ions were hyper-regulated with respect to the ambient water at 10 and 20‰ *S*. Hemolymph ion levels were 4.0, 2.0, 1.8, and 1.4 times the level in the ambient water at 10‰ *S* for K^+, Na^+, Mg^{2+}, and Cl , respectively. Significant quadratic effects of salinity treatment were only found on osmolality and K^+ concentration, indicating that these variables tend to be regulated independently of ambient sea-water concentration in the lower end of the salinity range used in this study. No differences due to sex of the hermit crab were observed in any aspect of osmoregulation reported here.

Hemolymph osmolality followed changes in ambient salinity during diurnal and semidiurnal cycles of fluctuating salinity (Fig. 1). The 30–10–30‰ *S* pattern elicited a 30% decrease in hemolymph osmolality (from control value of 30‰ to 10‰) during the diurnal cycle compared to 24% during the semidiurnal cycle. Similarly,

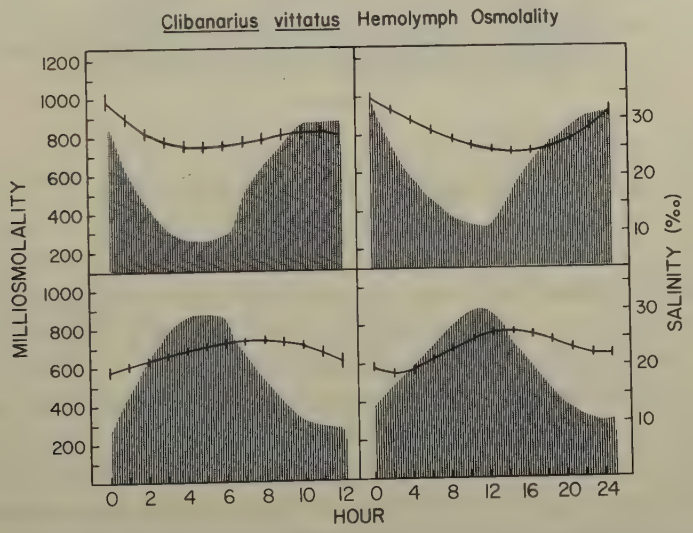


Fig. 1. Time course of changes in hemolymph osmolality (mOsm) for *Clibanarius vittatus* under fluctuating salinity conditions as shown by maximum R^2 improvement regression lines with 95% confidence limits: ambient sea-water salinity (‰) and osmolality (mOsm) is indicated by shading.

hemolymph osmolality increased to 140% of control during the 10–30–10‰ *S* diurnal cycle compared to a 125% increase during the semidiurnal cycle. Hemolymph osmolality remained hyperosmotic to ambient water throughout most of both 30–10–30‰ *S* patterns of fluctuating salinity and became iso- or slightly hypo-osmotic to the ambient water as salinity increased to 25‰ *S*.

No significant change ($P > 0.05$) occurred in per cent body water during either pattern or duration of salinity fluctuation. Water volume regulation was quite adequate in *C. vittatus* during short-term salinity fluctuations.

Changes in hemolymph sodium ion concentration during salinity fluctuation paralleled those of hemolymph osmolality and indicated that the duration of fluctuation is important (Fig. 2). During 10–30–10‰ *S* cycles, sodium increased to 137%

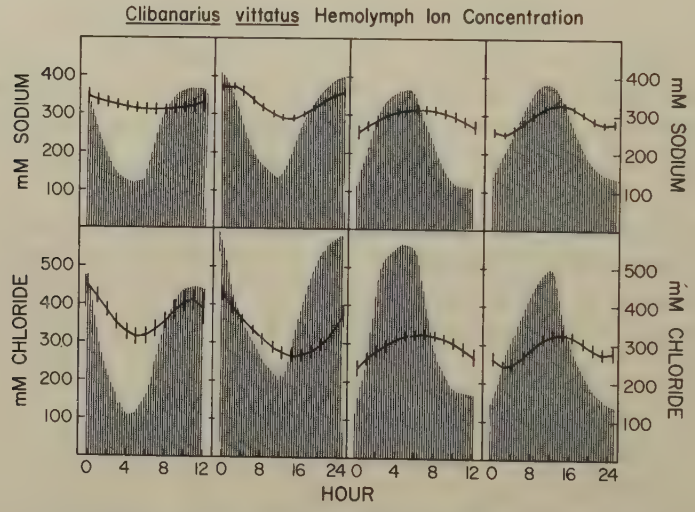


Fig. 2. Time course of changes in hemolymph concentrations (mM) of sodium and chloride for *Clibanarius vittatus* under fluctuating salinity conditions as shown by maximum R^2 improvement regression lines with 95% confidence limits: ambient sea-water specific ion concentrations (mM) are indicated by shading.

of control in the diurnal cycle and to 129% of control in the semidiurnal cycle, while during the 30–10–30‰ *S* pattern it decreased to 76% of control in the diurnal cycle and to 85% of control in the semidiurnal cycle.

Hemolymph chloride and magnesium concentrations deviated from control levels more than sodium during salinity fluctuations, particularly during the 30–10–30‰ *S* cycles (Figs. 2 and 3). Hemolymph chloride concentration declined to 62% of control during the diurnal cycle and to 70% of control during the semidiurnal cycle. Similarly, magnesium declined to 66% of control during the diurnal cycle and 70% of control during the semidiurnal cycle. Compared to sodium, the hemolymph concentration of chloride and magnesium lagged further behind the changing ionic

conditions of the ambient water to the extent of becoming extremely hypoionic for the latter portion of the 30–10–30‰ S experiment.

Hemolymph potassium was regulated hyperionically during all fluctuations, but followed changes of ambient water potassium only during diurnal cycles (Fig. 3). The time course of semidiurnal cycles possibly was too fast to allow the extracellular and intracellular potassium pools to establish an equilibrium; hence, potassium levels were unaltered.

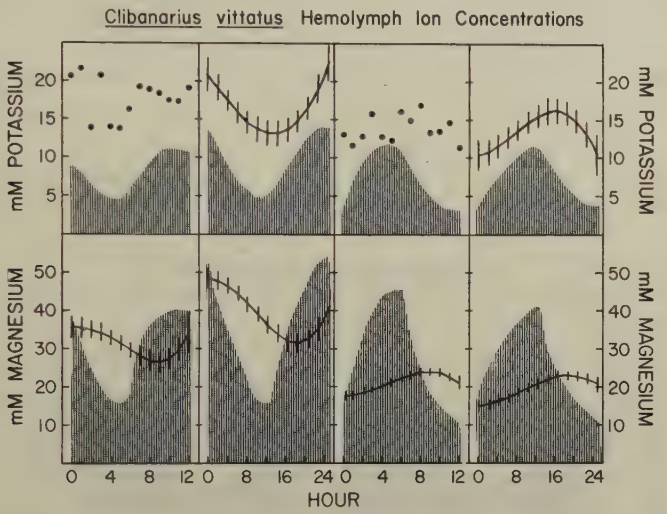


Fig. 3. Time course of changes in hemolymph concentrations (mM) of potassium and magnesium for *Clibanarius vittatus* under fluctuating salinity conditions as shown by maximum R^2 improvement regression lines with 95% confidence limits: ambient sea-water specific ion concentrations (mM) are indicated by shading.

In addition to work done with hermit crabs in their shells, we included groups of shell-less crabs during both semidiurnal cycles. Our intention was to determine whether the shell buffers the animal from fluctuating ambient salinity. Hemolymph osmolality, ions and per cent body water did not differ from that of crabs remaining in their shells during either fluctuation experiment. Maximum R-square improvement regressions were run for all of these variables on shell-less crabs. The models were virtually identical to those derived for crabs with shells.

OXYGEN CONSUMPTION

Oxygen consumption rates for crabs acclimated to constant salinities of 10, 20, and 30‰ S and 21 ± 1 °C are listed in Table II. Rates during the winter were significantly lower than those during summer at both 10 ($P < 0.001$, Student's t test) and 30‰ S ($P < 0.02$). Summer rates at 10 and 20‰ S, though not statistically

different from each other, are both significantly higher than the oxygen consumption rate at 30‰ S. The effect of salinity treatments on oxygen uptake during the summer are described by a quadratic response at the $P < 0.01$ level. However, the rates of oxygen consumption of crabs held at 10 and 30‰ S during the winter did not differ.

TABLE II

Oxygen consumption rates (μl $O_2 \cdot$ g dry wt$^{-1} \cdot$ h$^{-1} \pm$ SE) for *Clibanarius vittatus* acclimated to constant salinity during summer and winter at 21 ± 1 °C: n, sample size; values have been corrected for rate/weight relationships on basis of average sized crab of 0.7 g.

Salinity (‰)	O_2 uptake (summer)	O_2 uptake (winter)
30	423 ± 25 (16)	321 ± 32 (11)
20	576 ± 47 (10)	–
10	553 ± 21 (15)	301 ± 39 (9)

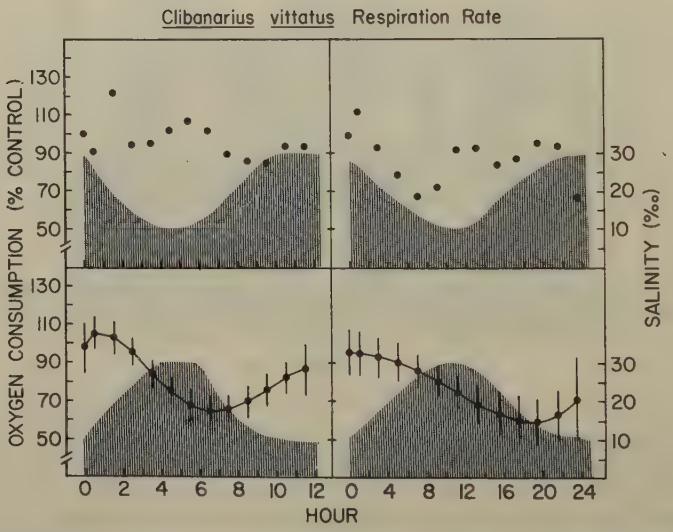


Fig. 4. Time course of normalized respiration rate for *Clibanarius vittatus* under fluctuating salinity conditions as shown by maximum R^2 improvement regression lines with 95% confidence limits: where no regression equation could be generated the normalized respiration rate is given as solid circles; ambient salinity (‰) is indicated by shading.

When crabs were subjected to salinity fluctuations, only the 10–30–10‰ S cycles elicited a significant change in oxygen consumption rate (Fig. 4). The rate declined with increasing salinity and returned towards control as salinity was returned to 10‰. The minimum oxygen uptake rates were 64% and 59% of control during the semidiurnal (summer crabs) and diurnal (winter crabs) fluctuation patterns, respectively. The minimal respiration rate lagged behind the fluctuating ambient

salinity. This time lag is more prominent during the diurnal fluctuation (minimum between hours 18.6 and 20.7 versus hours 7 and 8 in semidiurnal cycle), and is similar to results described above for chloride and magnesium concentration changes during similar ambient fluctuations.

TABLE III

Average hourly rate of oxygen consumption ($\mu l\ O_2 \cdot g\ dry\ wt^{-1} \cdot h^{-1} \pm SE$) for *Clibanarius vittatus* under steady-state and fluctuating salinity conditions: *n*, sample size; values have been corrected for rate–weight relationships on basis of average sized crab of 0.7 g; NS, nonsignificant at the 0.05 level.

Experiment	*n*	Control rate	$\bar{X}$ cycle rate	Student's *t* test
30–10–30 Semidiurnal	6	422 ± 28	281 ± 10	$P < 0.01$
10–30–10 Semidiurnal	6	590 ± 17	297 ± 14	$P < 0.001$
30–10–30 Diurnal	6	375 ± 39	312 ± 48	NS
10–30–10 Diurnal	4	264 ± 51	190 ± 29	NS

Table III shows the difference between the oxygen consumption rate during salinity fluctuation and the individual control rate for each fluctuation experiment. The control rates used here are those recorded prior to salinity fluctuation for the hermit crabs used in fluctuating conditions only. Both semidiurnal fluctuation patterns elicited a significant decline in oxygen consumption rate during the cycle. Mean cycle oxygen uptake rates were not significantly lower than control rates during either diurnal cycle ($P > 0.05$).

DISCUSSION

An important problem encountered by anomurans under salinity stress is body water regulation. Inability to tolerate osmotic alterations of the medium has been related to species differences (Davenport, 1972a; Bursey & Bonner, 1977) and intraspecifically to size (Davenport, 1972b). For the size range and experimental conditions in our study, *C. vittatus* acts as a near perfect water volume regulator. It has been shown to maintain uniform body water content over a range of 20‰ *S* (10–30‰) when held at the final salinity longer than 7 days, under diurnal fluctuations (20‰ in 10.4 h) and under semidiurnal fluctuations (20‰ in 4 h) in either direction. *C. vittatus* transferred directly from 34.5 to 10.5‰ *S* exhibited a weight gain of only 2.3 to 3.4% after 5 h (Fotheringham, 1975). Davenport (1972b, c) has suggested that hermit crabs achieve adequate volume regulation by adjusting urinary excretion volumes. If *C. vittatus* utilizes excretory processes to control water volume, it exhibits a great degree of control over excretory functions.

The hemolymph osmolality of *C. vittatus* follows the Type III adaptive response described by Vernberg & Vernberg (1972) to differing acclimation salinities; i.e., the body fluids are hyperosmotic to the medium below 25–28‰ *S* and isosmotic above.

The Type III pattern also holds for conditions of fluctuating salinity. Recently, the blue crab *Callinectes sapidus* (Rathbun), was reported to have a similar response under constant and fluctuating salinity regimes (Findley & Stickle, 1978). Tight control over hemolymph osmolality in stepwise acclimated crabs and those exposed to fluctuating salinity indicates the presence of anisosmotic extracellular regulation (Schoffeniels & Gilles, 1970), an adaptation which allows many euryhaline crustaceans to extend their zone of capacity adaptation with regard to salinity.

Hemolymph sodium and chloride concentrations in constant and fluctuating salinity, also indicative of this type of regulation, are similar to those reported for numerous brachyuran species (Kinne, 1971). Several researchers have recently sought to demonstrate the role of active transport systems in ion regulating crustaceans (Mantel, 1967; Towle *et al.*, 1976; Pequeux & Gilles, 1977). The more precise regulation of sodium compared to chloride in the hermit crabs under differing osmotic conditions is in agreement with the idea that sodium and chloride transport may be independent processes (Mantel, 1967).

Changes in integumental water permeability and net ion flux may play an important role during the initial stages of salinity fluctuation. Capen (1972) has shown the euryhaline crab, *Rhithropanopeus harrisii* (Gould), to be less permeable to water uptake, primarily through the gill chambers, in 10% sea water than 75% sea water. Crabs transferred directly from 75 to 10% sea water exhibited a measurable reduction in water uptake within 30 min, 50% of the change had occurred by 2 h and over 90% of full acclimation by 2 days. In our study, salinity fluctuations in either direction elicited greater changes in ion concentration during diurnal cycles than in semidiurnal cycles over the same salinity amplitude. Because changes in body water during salinity fluctuation were minimal, most of the changes in ion concentration were due to change in net flux of the respective ions. The faster rate of salinity change during semidiurnal than diurnal cycles of fluctuating salinity may trigger more rapid adjustments in the net flux of ions and water permeability. Alternatively, greater fluctuations in hemolymph ion concentration during diurnal cycles of fluctuating salinity may be due to a longer duration of altered ambient salinity. Towle *et al.* (1976) showed that Na^+/K^+-ATPase specific activity of *Callinectes sapidus* gills increased considerably within 2.5 h after transfer of crabs from 34 to 5‰. Increase in ion pump activity may be due to the amplitude of salinity change (or ion change) or to the rate of salinity change. Further experimentation will be necessary to distinguish between these potential causes for the observed differences in hemolymph ion fluctuation during diurnal and semidiurnal cycles of fluctuating salinity.

Hemolymph patterns of the divalent cation, magnesium, exhibited a longer lag period behind sea-water values during fluctuating salinity than observed for the other ions studied. This may reflect the larger hydrated diameter of Mg^{2+}, or be due to a difference in the route by which magnesium enters and leaves the hemolymph (renal) compared to sodium (gills) (Kinne, 1971).

Hemolymph magnesium levels are maintained higher than those reported for

most osmoregulating crustaceans (Gilles, 1975). Furthermore, the levels of Mg^{2+} in 20 and 10‰ *S* are ≈1.5 and 1.8 times the levels of magnesium in the ambient water, respectively. If Robertson's (1953) suggestion that blood Mg^{2+} levels are inversely proportional to locomotor activity in decapods is true, then *Clibanarius vittatus* should be a very sedentary species. Many laboratory and field behavioral observations, however, indicate that this is not the case for *C. vittatus*.

The shell inhabited by *C. vittatus* is not important in the maintenance of hemolymph-ambient water osmotic gradients. This observation differs from results reported by Shumway (1978) for the osmoconforming hermit crab *Pagurus bernhardus* (L.). Shell-dwelling hermit crabs in her study exhibited greater control of hemolymph osmolality and tissue water content than shell-less counterparts under conditions of a 32–16–32‰ *S* semidiurnal fluctuation. We observed no difference in the osmotic response of shell-dwelling and shell-less *Clibanarius vittatus* to fluctuating salinity. *Pagurus bernhardus* also displayed increased locomotor activity followed by withdrawal into the shell with declining salinity, whereas we observed no behavioral variation in *Clibanarius vittatus*. Such differences in behavior between the two species of hermit crabs are probably a reflection of the differences between an osmoregulator and osmoconformer.

Marked seasonal differences in oxygen consumption were recorded at the same acclimation temperature. Winter acclimated crabs had a significantly lower rate of oxygen consumption at both 10 and 30‰ *S* than summer acclimated crabs. Similar results have been reported by Dehnel (1961) for *Hemigrapsus nudus* (Dana) and *H. oregonensis* (Dana). Engel & Eggert (1974) and Findley *et al.* (1978), on the other hand, have shown that excised gill tissue and whole *Callinectes sapidus* respire at a lower rate during the summer. Winter hermit crabs at 10 and 30‰ *S* were also shown in our experiments to respire at the same rate, whereas the rates at 10 and 20‰ *S* were significantly higher than those at 30‰ *S* for summer animals. The latter observation is generally expected for osmoregulating crustaceans (Kinne, 1971). Lack of a salinity effect with particular season has also been reported for *C. sapidus* (Laird & Haefner, 1976). The possibility that these observations are related to seasonal changes in osmoregulatory ability in the crabs cannot be eliminated. Seasonal differences in osmoregulation of *Hemigrapsus nudus* and *H. oregonensis* have been documented by Dehnel (1962). Hemolymph chloride (Dehnel, 1966) and calcium were more effectively regulated by winter crabs, but sodium, potassium and magnesium did not show consistent trends (Dehnel & Carefoot, 1965). All ionic and osmoregulatory experiments in the present study were completed during the summer. Most members of this species from littoral populations are known to migrate to the sublittoral zone for 2–3 months during the winter season (Fotheringham, 1975).

Semidiurnal and diurnal cycles of 10–30–10‰ fluctuating salinity elicited an oxygen consumption pattern similar to that recently reported for a diurnal cycle in *Callinectes sapidus* (Findley *et al.*, 1978). Normalized rates for 30–10–30‰ *S* cycles

could not be modelled, indicating that exposure to fluctuation after acclimation to 30‰ *S* has less effect on time course changes of oxygen consumption in hermit crabs than exposure after acclimation to 10‰ *S*. As in osmoregulation, it is apparent that salinity cycles fluctuating in opposite directions result in different patterns of oxygen uptake.

Oxygen consumption rate was significantly lower than controls during semidiurnal cycles but not during diurnal cycles. This observation can be attributed either to changing seasonal responses or to temporal physiological adjustments. Seasonal changes in response to fluctuating salinity have not been previously reported.

Some have attributed oxygen consumption changes in crustaceans under osmotic stress to the cost of osmoregulation (see Kinne, 1971; Taylor *et al.*, 1977). In such a case the high oxygen uptake rate at low acclimation salinity is expected. However, with cycling salinity, average oxygen consumption rates should be lower than control during a 10–30–10‰ *S* pattern due to ambient osmolality approaching the hemolymph isosmotic point and higher during a 30 10–30‰ *S* pattern (Table III). This is not the case as both cycles elicited a decline in oxygen consumption which was more evident during semidiurnal, or faster, rate of salinity change. Since there was no observed change in locomotor activity during fluctuating salinity, processes which regulate hemolymph and ionic composition within narrowly defined limits may account for the changes in oxygen uptake observed.

Possibly the more gradual salinity change in the diurnal cycle, which causes ion and amino acid fluxes to bring the cells into closer equilibrium with the hemolymph and ambient water, can account for the lack of a significant difference in cycle versus control oxygen consumption rate. Compensatory metabolic adjustments would be operative for a longer duration during diurnal cycles. Another explanation for lower aerobic metabolism in semidiurnal, compared to diurnal, cycles is that anaerobic metabolism predominates in a short term salinity fluctuation, as is expected under normal hyperosmotic conditions (Schoffeniels, 1976). Laird & Haefner (1976) attributed a lack of salinity effect on oxygen consumption of *C. sapidus* to be due either to anaerobic metabolism or an undetectable metabolic cost of osmoregulation. Anaerobic metabolism has not been measured in the present study, but should be considered in future research on responses of invertebrates to salinity fluctuations. This may explain some of the discrepancies which occur when considering seasons, temporal conditions of salinity change, and inverse cycles of salinity change.

Knowledge of the changes which occur in the cardiovascular and ventilatory system is also essential to our understanding of adjustments in crustacean oxygen delivery under conditions of fluctuating salinity. Taylor (1977) has determined that oxygen consumption, ventilation volume, and cardiac output respond in an inverse manner to short-term salinity change in *Carcinus maenas* (L.). We are currently taking a more extensive look at the oxygen delivery system of an osmoregulating decapod, *Callinectes sapidus*, in constant and fluctuating salinity regimes.

Acknowledgements

We gratefully acknowledge Drs. B. L. Bayne and S. E. Shumway for constructively criticizing this manuscript. T. D. Sabourin is the recipient of a fellowship from the Petroleum Refiners Environmental Council of Louisiana.

References

Barrett, B. B., 1971. Phase II, hydrology. In, *Cooperative Gulf of Mexico estuarine inventory and study, Louisiana*. Louisiana Wildlife and Fisheries Commission, New Orleans, Louisiana, pp. 9–130.

Bryan, G. W., 1965. Ionic regulation in the squat lobster *Galathea squamifera* with special reference to the relationship between potassium metabolism and the accumulation of radioactive caesium. *J. mar. biol. Ass. U.K.*, Vol. 45, pp. 97–113.

Bursey, C. R. & E. E. Bonner, 1977. Osmotic regulation and salinity tolerance of the mole crab, *Emerita talpoida* (Say). *Comp. Biochem. Physiol.*, Vol. 57A, pp. 207–210.

Capen, R. L., 1972. Studies of water uptake in the euryhaline crab, *Rhithropanopeus harrisii*. *J. exp. Zool.*, Vol. 182, pp. 307–320.

Davenport, J., 1972a. Salinity tolerance and preference in the porcelain crabs, *Porcellana platycheles* and *Porcellana longicornis*. *Mar. Behav. Physiol.*, Vol. 1, pp. 123–138.

Davenport, J., 1972b. Effects of size upon salinity tolerance and volume regulation in the hermit crab *Pagurus bernhardus*. *Mar. Biol.*, Vol. 17, pp. 222–227.

Davenport, J., 1972c. Volume changes shown by some littoral anomuran crustacea. *J. mar. biol. Ass. U.K.*, Vol. 52, pp. 863–867.

Dehnel, P. A., 1960. Effect of temperature and salinity on the oxygen consumption of two intertidal crabs. *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 118, pp. 215–249.

Dehnel, P. A., 1962. Aspects of osmoregulation in two species of intertidal crabs. *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 122, pp. 208–227.

Dehnel, P. A., 1966. Chloride regulation in the crab *Hemigrapsus nudus*. *Physiol. Zool.*, Vol. 39, pp. 259–265.

Dehnel, P. A. & T. H. Carefoot, 1965. Ion regulation in two species of intertidal crabs. *Comp. Biochem. Physiol.*, Vol. 15, pp. 377–397.

Engel, D. W. & L. D. Eggert, 1974. The effect of salinity and sex on the respiration rates of excised gills of the blue crab, *Callinectes sapidus*. *Comp. Biochem. Physiol.*, Vol. 47A, pp. 1005–1012.

Findley, A. M., B. W. Belisle & W. B. Stickle, 1978. Effects of salinity fluctuations on the respiration rate of the southern oyster drill *Thais haemastoma* and the blue crab *Callinectes sapidus*. *Mar. Biol.*, Vol. 49, pp. 59–67.

Findley, A. M. & W. B. Stickle, 1978. Effects of salinity fluctuation on the hemolymph composition of the blue crab *Callinectes sapidus*. *Mar. Biol.*, Vol. 46, pp. 9–15.

Fotheringham, N., 1975. Structure of seasonal migrations of the littoral hermit crab *Clibanarius vittatus* (Bosc). *J. exp. mar. Biol. Ecol.*, Vol. 18, pp. 47–53.

Gilles, R., 1975. Mechanisms of ion and osmoregulation. In, *Marine ecology, Vol. 2, Part 1*, edited by O. Kinne, John Wiley and Sons, London, pp. 259–347.

Hewatt, W. G., 1951. Salinity studies in Louisiana coastal embayments west of the Mississippi River. Final Report of Project Nine. Texas A & M Research Foundation, College Station, Texas, 32 pp.

Kinne, O., 1971. Salinity – animals – invertebrates. In, *Marine ecology, Vol. 1, Part 2*, edited by O. Kinne, Wiley-Interscience, London, pp. 821–995.

Laird, C. E. & P. A. Haefner, Jr., 1976. Effects of intrinsic and environmental factors on oxygen consumption in the blue crab, *Callinectes sapidus* Rathbun. *J. exp. mar. Biol. Ecol.*, Vol. 22, pp. 171–178.

Lockwood, A. P. M., 1962. The osmoregulation of Crustacea. *Biol. Rev.*, Vol. 37, pp. 257–305.

Mangum, C. P. & D. W. Towle, 1977. Physiological adaptation to unstable environments. *Am. Scient.*, Vol. 65, pp. 67–75.

MANTEL, L. H., 1967. Asymmetry potentials, metabolism and sodium fluxes in gills of the blue crab, *Callinectes sapidus*. *Comp. Biochem. Physiol.*, Vol. 20, pp. 743–753.

NATIONAL OCEAN SURVEY, 1977. *Tide tables*. East Coast of North and South America, U. S. Department of Commerce, National Oceanic and Atmospheric Administration, Washington, D. C., 288 pp.

PEQUEUX, A. & R. GILLES, 1977. Osmoregulation of the Chinese crab *Eriocheir sinensis* as related to the activity of the (Na^+, K^+) ATPase. *Archs int. Physiol. Biochim.*, Vol. 85, pp. 426–427.

ROBERTSON, J. D., 1953. Further studies on ionic regulation in marine invertebrates. *J. exp. Biol.*, Vol. 26, pp. 182–200.

SCHOFFENIELS, E., 1976. Adaptations with respect to salinity. *Biochem. Soc. Symp.*, Vol. 41, pp. 179–204.

SCHOFFENIELS, E. & R. GILLES, 1970. Osmoregulation in aquatic arthropods. In, *Chemical zoology, Vol. 5, Arthropoda, Part A*, edited by M. Florkin & B. T. Scheer, Academic Press, New York, pp. 255–286.

SERVICE, J., 1972. *A users guide to the statistical analysis system*. North Carolina State University Student Supply Store, Raleigh, North Carolina, 260 pp.

SHUMWAY, S. E., 1978. Osmotic balance and respiration in the hermit crab, *Pagurus bernhardus*, exposed to fluctuating salinities. *J. mar. biol., Ass. U. K.*, Vol. 58, pp. 869–876.

SMITH, R. I., 1976. Apparent water-permeability variation and water exchange in crustaceans and annelids. *Perspect. exp. Biol.*, Vol. 1, pp. 17–24.

SPAARGAREN, D. H., 1975. Changes in permeability in the shore crab, *Carcinus maenas* (L.), as a response to salinity. *Comp. Biochem., Physiol.*, Vol. 51A, pp. 549–552.

STEEL, R. G. D. & D. H. TORRIE, 1960. *Principles and procedures of statistics*. McGraw-Hill, New York, 481 pp.

STICKLE, W. B. & T. D. SABOURIN, 1979. Effects of salinity on the respiration and heart rate of the common mussel, *Mytilus edulis* L., and the black chiton, *Katherina tunicata* (Wood). *J. exp. mar. Biol. Ecol.*, Vol. 41, pp. 257–268.

STICKLE, W. B. & T. W. HOWEY, 1975. Effects of tidal fluctuations of salinity on hemolymph composition of the Southern oyster drill *Thais haemastoma*. *Mar. Biol.*, Vol. 33, pp. 309–322.

TAYLOR, A. C., 1977. The respiratory responses of *Carcinus maenas* (L.) to changes in environmental salinity. *J. exp. mar. Biol. Ecol.*, Vol. 29, pp. 197–210.

TAYLOR, E. W., P. J. BUTLER & A. AL-WASSIA, 1977. The effect of a decrease in salinity on respiration, osmoregulation and activity in the shore crab, *Carcinus maenas* at different acclimation temperatures. *J. comp. Physiol.*, Vol. 119, pp. 155–170.

TOWLE, D. W., G. E. PALMER & J. L. HARRIS III, 1976. Role of gill $Na^+ + K^+$-dependent ATPase in acclimation of blue crabs to low salinity. *J. exp. Zool.*, Vol. 196, pp. 315–322.

VERNBERG, W. B. & F. J. VERNBERG, 1972. *Environmental physiology of marine animals*. Springer-Verlag, New York, 346 pp.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 255–277
© Elsevier/North-Holland Biomedical Press

OXYGEN UPTAKE, THE CIRCULATORY SYSTEM, AND HAEMOGLOBIN FUNCTION IN THE INTERTIDAL POLYCHAETE *TEREBELLA HAPLOCHAETA* (Ehlers)

R. M. G. Wells, P. J. Jarvis
Department of Zoology, University of Auckland, Auckland, New Zealand

and

S. E. Shumway
Portobello Marine Laboratory, University of Otago, Portobello, New Zealand

Abstract: The intertidal polychaete *Terebella haplochaeta* (Ehlers) shows a high degree of oxyregulation in declining pO_2 when confined to its burrow at low tide. This response is achieved by a number of adaptations to the respiratory system. The worm ventilates its burrow in a headward direction by rhythmical contractions of the body. The rate of these pulsations increases at low pO_2 and assists the circulation of the coelomic and vascular fluids. Haemoglobin in the vessels has a high affinity for oxygen and a sigmoidal equilibrium curve. Both the shape and position of the oxygen-binding curve are sensitive to changes in pH, pCO_2, and temperature in a way that suggests augmentation of oxygen delivery at low tide. The concentration of haemoglobin in the vessels is high and is further raised following warm acclimation, presumably to meet an increase in oxygen demand. The ultrastructure of the gills and blood vessels indicates a design for function at low oxygen tensions where diffusion distances must be short and surface areas large in order to enhance the rate of diffusion of oxygen from the near environment.

Introduction

The past ten years has seen a growing interest in the respiratory adaptations of marine organisms, particularly with regard to the Polychaeta, Gastropoda, and Crustacea. Studies which have focused on intertidal invertebrates have had to take into account the transition from aquatic to aerial conditions occurring with each phase of the tide. Several reviewers have drawn attention to the difficulties in interpreting the interaction of environmental and organismic factors in the complex intertidal zone (see Mangum, 1970, 1976a, b; Mangum & Van Winkle, 1973; Newell, 1973; Weber, 1978a).

Many intertidal animals possess a respiratory pigment, and among those that do, the Polychaeta frequently have haemoglobin dissolved in the plasma of the vascular system or a cellular haemoglobin contained in the coelom. The oxygen-binding properties of these pigments are now known to be closely correlated with the availability of oxygen in the near environment, even though precise field measurements are often lacking. The recent interest in this aspect of respiratory behaviour is

reflected in the reviews by Mangum (1970, 1976a, b), Wells & Dales (1976), Weber (1978b), and Chung & Ellerton (1979).

Unfortunately, there have been few attempts to describe the adaptations of a whole respiratory system by taking into account the interactions between the parts of the system at a molecular level (the haemoglobin), the systemic level (the circulation), and the organismic level. A notable exception has been the synthetic approach of C. P. Mangum and her colleagues (e.g. Mangum *et al.*, 1975; Mangum, 1976a, b). In the present study, we have attempted to consider some of the components which comprise the respiratory system in the intertidal polychaete *Terebella haplochaeta* (Ehlers).

Material and Methods

Collection of material

Terebella haplochaeta were collected from burrows in sandstone, in the intertidal zone at Cheltenham Beach, Auckland, New Zealand. Whenever possible, individuals were used immediately on return to the laboratory. Animals collected for studies of oxygen uptake were flown to the Portobello Marine Laboratory where they were kept for at least 1 wk prior to experimentation. All animals were maintained in aerated sea water at 16–18 °C.

Collection of blood

Animals were blotted and an incision made on the anterior dorsal surface with care being taken to avoid puncturing the large dorsal blood vessel. Coelomic fluid was collected or discarded. The worm was then rinsed with filtered sea water and again blotted. The dorsal blood vessel was cut and the blood collected into 5-μl graduated glass capillary tubes.

Laboratory reproduction of the habitat

Worms were kept in a dark aquarium with fresh, aerated sea water and when provided with sediment from the natural habitat, built tubes similar to those found on the shore. As one side of the tube comprised the aquarium wall the worms were plainly visible. All observations were made in darkness using an infrared light.

Sea water was siphoned out over a 4-h period (ebbing tide), the burrows were left uncovered for 2 h (slack tide), and then flooded with aerated sea water to simulate a tidal cycle. Samples of burrow water (0.5 ml) were taken into a glass syringe and the oxygen content measured using a Radiometer E5046 electrode. Five worms were introduced into Pasteur pipettes and the animals' irrigatory behaviour was observed in these transparent burrows. This assumes that in nature the tubes are watertight. While we lack direct evidence for this assertion, *T. haplochaeta* does construct

permanent, mucus-lined tubes. Gill colouration was observed over an outgoing tide and estimations of oxyhaemoglobin in the gills were made by comparison with known percentages of human oxyhaemoglobin prepared by anaerobically mixing various ratios of oxy : deoxy haemoglobin in glass capillaries.

When all the water had been siphoned from an aquarium (low tide), estimations of the volume of water trapped in burrows were obtained by extraction into graduated glass syringes.

Irrigatory waves progressing anteriorly up the worm's body were observed during the simulated tidal cycle. Twenty animals were used at oxygen concentrations ranging from 100% to 14.2% saturation. These worms were kept in sealed syringes having an initial capacity of 4 ml of fully oxygenated sea water at 20 °C. The number of waves $\cdot$ min^{-1} were counted every 5 min. The pO_2 of the water was measured with the oxygen electrode used to determine oxygen in burrow water. The end of the control syringe was left open in a beaker of oxygenated sea water in the same water bath.

Histology, morphology, and anatomy

Estimations of body, gill, and blood vessel wall thicknesses were made from slides of sectioned worms using a micrometer eye piece fitted to a compound light microscope. Animals were narcotized using menthol in sea water and then fixed in Bouin's solution for 24 h. Whole worms were embedded in paraffin wax and sectioned with a Jung Rotary Microtome at 5-μm intervals. Every sixth section of the anterior half of a worm was taken for inspection.

Gills were examined for the possible presence and arrangement of capillaries by taking every section. Mallory and Heidenhain's rapid one-step method of staining was used (Humason, 1972).

The fate of the major blood vessels was traced by dissecting freshly collected worms and by examining sectioned worms. Estimation of the volume and surface area of the entire animal was more difficult. Previous work of this nature on annelids has seen the gills treated as cylinders (Mangum *et al.*, 1975). The gills of *T. haplochaeta* are not cylinders and any calculations are, therefore, only rough estimations. For this reason, measurement of each "branchlet" of the gill was taken with a micrometer eye piece and the data used for volume calculation. Surface area calculations of gills also give only approximate estimates and, therefore, finer details of the gill surfaces were noted by means of the compound microscope but were not used in calculation.

Gills from narcotized animals were amputated and fixed with 2% glutaraldehyde in 0.2% cacodylate buffer (1150 mOsm). The tissue was stored in the buffer at 4 °C, then later dehydrated, and dried under CO_2 in a Polaron E3000 Critical Point Drier before examination under a Jeol JSM U3 scanning electron microscope. Prior to examination under the transmission electron microscope (Philips EM301) gills were

fixed in 1% glutaraldehyde (1100 mOsm) for 1–2 h. The tissue was stored in phosphate buffer for 24 h and post-fixed in 4% OsO_4 for 1 h, dehydrated in alcohol and embedded in 812 Epon. Sections were cut with a Sorvall MT-1 ultramicrotome and stained with 4% uranyl acetate for 3 min and lead citrate for 30 s (Venable & Coggeshall, 1965).

Oxygen uptake by whole animals

Oxygen uptake was monitored using a Radiometer E5046 electrode connected through a Radiometer PHM 71 meter to a Smith's Servoscribe chart recorder as described previously (Crisp *et al.*, 1978). Worms of ≈ 1 g wet wt were allowed to deplete the oxygen supply within a 120-ml respiratory chamber.

Spectrophotometric analysis

Samples of vascular blood and coelomic fluid were diluted with distilled water and scanned from 240–700 nm using a Unicam SP1750 recording spectrophotometer. Absorption maxima from the vascular pigment were similar to those obtained from other invertebrate haemoglobins (Chung & Ellerton, 1979) and confirmed the presence of haemoglobin in *T. haplochaeta*. Unlike other terebellids (Wells & Dales, 1975; Mangum *et al.*, 1975), no pigment was found in the coelomic cells of *T. haplochaeta*.

The concentration of haemoglobin was measured on a haem basis using the cyanmethaemoglobin derivative (Dacie & Lewis, 1975). Two μl blood were diluted with 4 ml of Drabkin's reagent and the absorbance measured at 540 nm in a 1-cm cuvette. A population of *T. haplochaeta* was sampled for haemoglobin concentration over two winters and one summer season in order to estimate possible changes in the oxygen-carrying capacity of the pigment.

Blood and coelomic fluid estimation

Coelomic fluid volumes were estimated gravimetrically by draining the coelomic contents; vascular blood volumes were estimated by comparing haemoglobin concentration in whole vascular fluid with that in whole, lacerated worms. The contribution from haemopoietic tissue and myoglobin was assumed to be negligible.

Carbonic anhydrase activity

Coelomic fluid, vascular blood, and various tissue extracts were tested for the presence of carbonic anhydrase by estimating their effect on the rate of carbon dioxide hydration (Wells, 1973).

Oxygen-binding studies

The reactions of haemoglobin with oxygen at various partial pressures of oxygen were measured using an Aminco Hem-O-Scan analyser as follows. A thin blood film was prepared from 2 μl of whole blood on a transparent coverslip and then placed in an optical path which photometrically monitored the percentage saturation. An O_2-electrode monitored the pO_2 of a progressively oxygenated gas mixture passing over the blood film in the humidified optical chamber. Saturation and pO_2 were recorded simultaneously on the Y and X channels of an X-Y recorder to produce a continuous oxygen equilibrium curve in $\approx$15 min. Whole blood curves were obtained at various temperatures and pCO_2, and for haemoglobin solutions buffered to constant pH with 0.05 M Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid) buffers. Hepes was selected because its pH is relatively insensitive to temperature, it has a negligible effect on ions other than H , and its pK is appropriate to the pH range 6.5–8.5.

Results

TIDAL ACTIVITY

The burrows of *T. haplochaeta* consist of sand grains cemented together by mucus secreted from the worms and the tubes are easily accessible to water when covered by the tide. Thus at high tide the worms are not likely to be isolated in pockets of water having low oxygen tensions as is the case with a number of other crevice-dwelling organisms (Menzies, 1968). The pO_2 of the overlying water column in the

Table I

Relationship between state of "tide" on an artificial beach, gill colour, ventilatory contractions, and oxygen content of water in the tubes of *T. haplochaeta*: contraction rates measured over 5 min and nearest whole number recorded; volume of burrow water when tide out, 0.2 to 0.4 ml; observations from five worms at 20 °C.

State of "tide"	Relative gill colour (1-deoxyHb) (4–oxyHb)	Contraction rate (waves · min^{-1})	Oxygen in burrow water (% saturation)
Fully in	4	6	98.0
1 h (tide ebbing)	4	6	94.1
2 h (tide ebbing)	4	8	91.3
3 h (tide ebbing)	3	8	90.6
Fully out	3	15	63.2
1 h out	2	9	40.2
2 h out	1	8	30.6
Tide in	3	7	80.2
0.5 h in	4	6	96.0

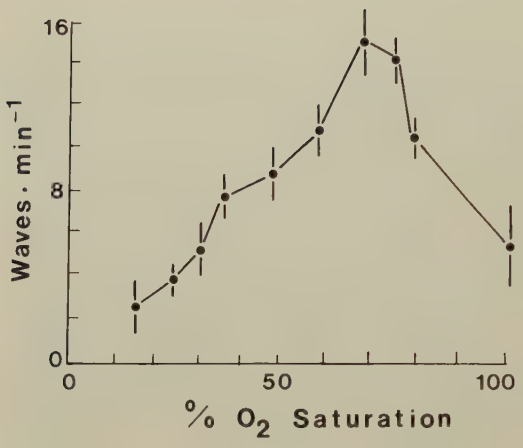


Fig. 1 (above). The effect of oxygen content of burrow water on ventilatory contractions (waves · min^{-1}) in *T. haplochaeta* acclimated to 20 °C: error bars show 95% confidence intervals.

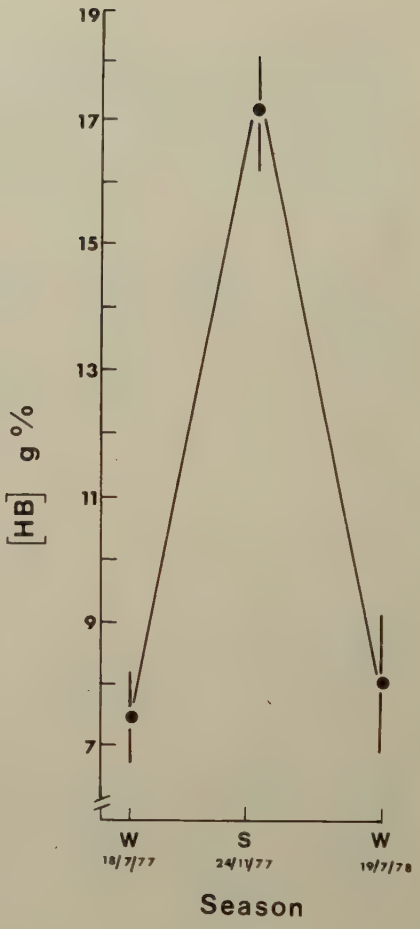


Fig. 2 (right). The relationship between the austral seasons and haemoglobin concentrations in the vascular fluid of *T. haplochaeta*: mean monthly air temperatures were 12.8 °C (18.7.77), 24.3 °C (24.11.77), 13.1 °C (19.7.78); error bars show 95% confidence intervals for 10 worms in each group.

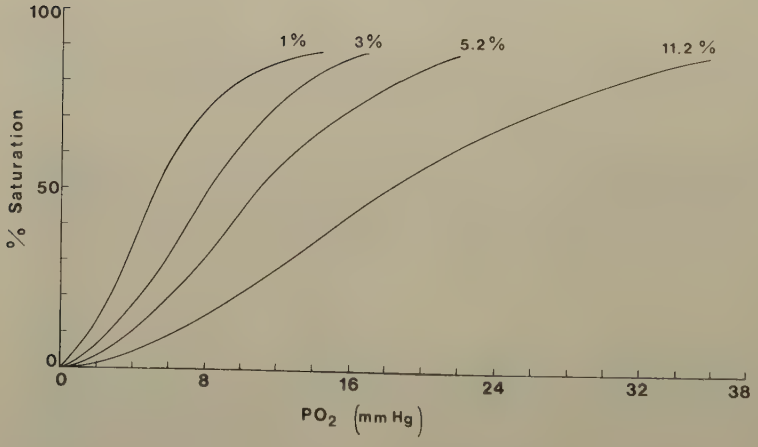


Fig. 3. Oxygen equilibrium curves of whole blood from *T. haplochaeta* at 20 °C equilibrated with 1–11.2% CO_2

washed intertidal zone is close to air saturation but when the tide recedes, the worms are deprived of a renewable supply of oxygenated water and remain trapped in the burrows in a small volume of water (0.2–0.4 ml).

Fig. 1 shows the relation of the rate of the body contractions to the oxygen tension of the overlying sea water. It is clear that at high oxygen tensions the ventilation rate is low (≈ 6 contractions $\cdot$ min^{-1}) and the rate increases with declining oxygen attaining a maximum of 15 contractions $\cdot$ min^{-1} at 70% saturation. Below this tension, the rate falls, but is still higher than at normoxic tensions, down to $\approx 40\%$ oxygen saturation. At high oxygen tensions it is unnecessary to maintain a vigorous ventilatory stream in order to maintain an adequate extraction of oxygen.

Table I summarizes the relative state of blood oxygenation and body contractions in *T. haplochaeta* as a function of the pO_2 of the burrow water during a single tidal cycle. The state of oxygenation of the blood, judged by the colour observed in the gills, follows the pattern of decreasing pO_2 in the burrow water. At high oxygen pressures the blood was fully oxygenated and appeared a scarlet colour. As the pO_2 of the burrow water decreased, the blood assumed a darker colour and appeared to be deoxygenated after 2 h emersion at $\approx 30\%$ saturation of oxygen. The blood within the gills lightened in colour almost immediately on return to oxygenated sea water and immersion of the burrows. It is worth noting, however, that Mangum (1976c) found that samples taken from a ventilated stream tended to over-estimate the amount of dissolved oxygen. While this might also be the case in the present study, the trend in deoxygenation of vascular blood paralleled the falling oxygen tensions as did the rate of ventilatory contractions.

HAEMOGLOBIN

The concentration of haemoglobin in the circulatory system of *T. haplochaeta* varies seasonally and maximum values were obtained in the summer (Fig. 2). A consequence of this is a seasonal shift in the oxygen-carrying capacity of the blood. Assuming that 1 g haem combines with 1.34 ml oxygen (Antonini & Brunori, 1971), then the blood may transport 23.1 vols oxygen per 100 ml blood during the summer, but only 10.3 vols % in winter. It is, however, not sufficient to have enough capacity because the blood must be able to give up as much of its bound oxygen as is demanded by the tissues. In the suite of haemoglobin-oxygen equilibrium curves in Fig. 3 it is evident that the affinity of *T. haplochaeta* haemoglobin decreases in response to a rise in carbon dioxide.

Apart from its effect on $P50$, pH has an influence on the shape of the curve as described by Hill's (1910) sigmoidal coefficient, n. The data in Fig. 4 show that in the absence of carbon dioxide, the equilibrium becomes less sigmoidal at low pH and at pH 6.8 the curve assumes a hyperbolic shape as indicated by an n value of 1.0. The equilibrium is also sensitive to temperature, the $P50$ rising with increased

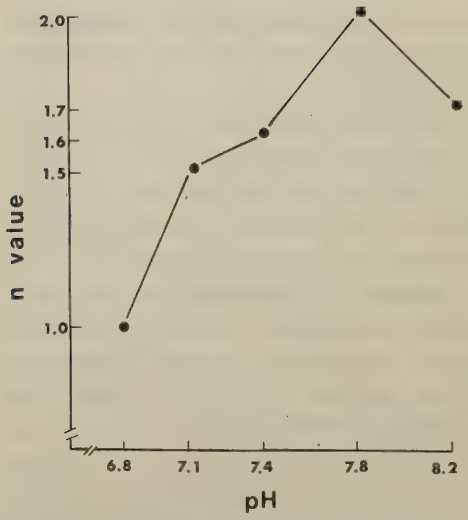


Fig. 4. Plot of the sigmoidal coefficient, *n*, from the haemoglobin equilibrium data obtained from blood buffered to various pH values and in the absence of carbon dioxide 20 °C.

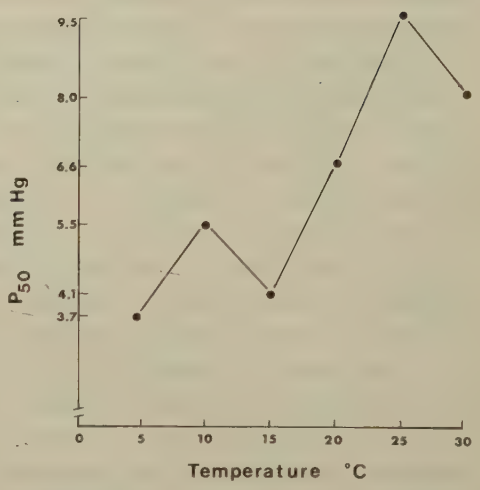


Fig. 5. The effect of temperature on oxygen affinity ($P50$) from the vascular blood of *T. haplochaeta* measured by duplicate points at 20 °C and buffered to pH 7.4.

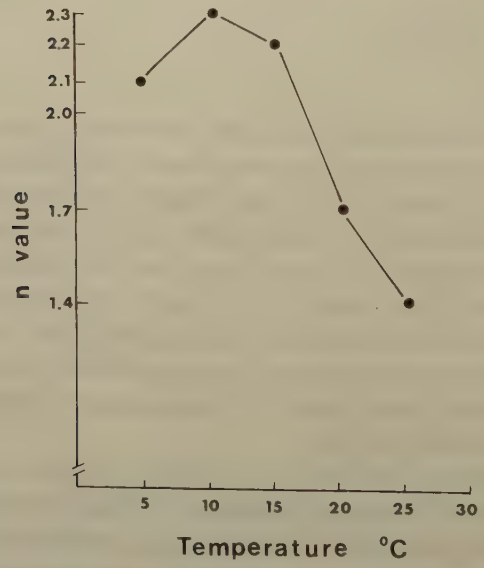


Fig. 6. Plot of the sigmoidal coefficient, *n*, of *T. haplochaeta* haemoglobin buffered to pH 7.4 at various temperatures.

temperature over the range 5–30 °C (Fig. 5). Deviations from linearity were assumed to be the result of sampling errors. The overall decrease in affinity with temperature is related to the exothermic nature of the oxygenation reaction and the quantitative relationship is given by the Van 't Hoff equation,

$$\Delta H^\circ = 19.159 \frac{T_1 T_2}{T_2 - T_1} \log \frac{P50_1}{P50_2} \text{ J} \cdot \text{mol}^{-1},$$

where ΔH° = heat of oxygenation, T_1, T_2 = lower and higher temperatures in °K, and $P50_1$, $P50_2$ = half saturation pO_2 at T_1 and T_2. Using a regression slope from the data in Fig. 5, ΔH° for *T. haplochaeta* haemoglobin was -21.6 kJ · mol^{-1} which falls within the wide range of -7.5 to -50.2 kJ · mol^{-1} reported for many haemoglobins from ectothermic animals (Rossi-Fanelli *et al.*, 1964).

Aside from oxygen affinity, temperature exerts an effect on the sigmoidal shape of the equilibrium (Fig. 6).

LOCATION AND ACTIVITY OF CARBONIC ANHYDRASE

Using arbitrary units of activity (see Wells, 1973), a high rate of catalysis was detected by duplicate determination in the vascular blood of *T. haplochaeta*, but not in the coelomic fluid. Gill and body wall tissues exhibited minimal activity, probably no more than could be accounted for by extracellular plasma arising from the circulation.

CIRCULATORY SYSTEM

Blood is pumped through the vascular system of *T. haplochaeta* by contractile waves of the coelomic fluid and pulsatile expansion of the large dorsal vessel and gills. The dorsal vessel (Fig. 7) is the most prominent vessel and is sometimes referred to as the heart. This vessel divides anteriorly into three: two afferent branchials and one supra-oesophageal which connects at the other end with the peri-oesophageal ring after running along the side of the oesophagus (Fig. 8). The efferent branchials return from the gills and join to form the ventral vessel, which runs the entire length of the body giving off a series of ventral ring vessels at the gut region. Before joining, however, the efferent branchials give rise to a vessel which divides and runs to the nephridia and mucus glands. Blood from these regions is collected and returned through a vessel which connects to the peri-oesophageal ring. Again, at the level of the ventral ring vessels, these are seen to be interconnected by loops, joining to form a lateral vessel. From the lateral vessel arises a series of dorsal ring vessels which pass into the gut plexus. The gut plexus drains into subintestinals and these in turn run forward to the peri-oesophageal vessel which gives rise to the dorsal vessel and oesophageal vessels.

Deoxygenated blood, or blood which has already circulated, is collected at the peri-oesophageal vessel and returned to the afferent branchials via the dorsal and supra-oesophageal vessels. Newly oxygenated blood is passed from the gills into the ventral vessel. This ventral vessel gives rise to a subneural vessel just before the level of the peri-oesophageal vessel, and runs the remaining length of the body in conjunction with the ventral nerve cord.

As oxygen is obtained only by diffusion from water across a physical barrier it is necessary to have some idea of the thicknesses of tissues across which the oxygen must diffuse. Assuming that the gills are the principal sites of oxygen uptake, it

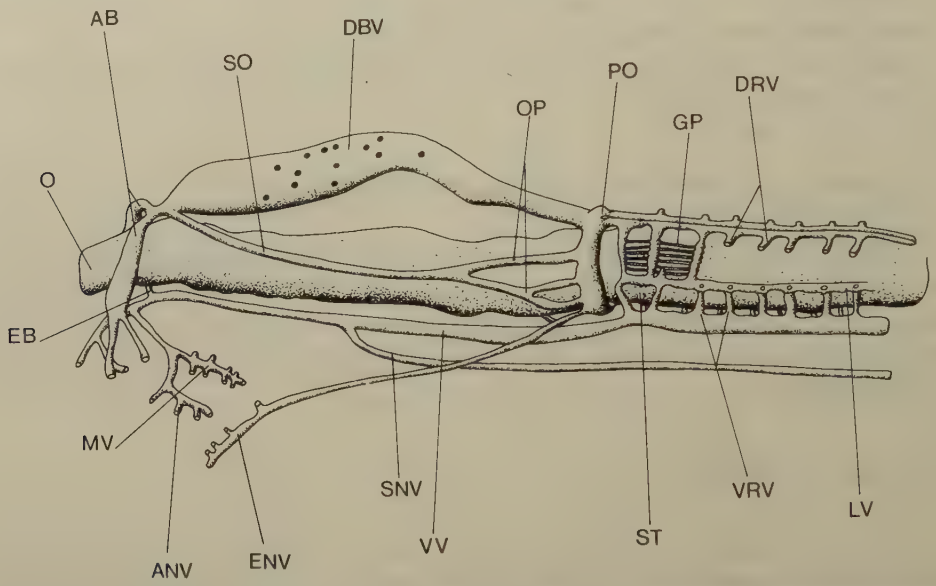


Fig. 7. Diagrammatic representation of the circulatory system in *T. haplochaeta* ($\approx \times 17$) indicating the major blood vessels when viewed from the lateral aspect: O, oesophagus; AB, afferent branchial; SO, supra-oesophageal; DBV, dorsal blood vessel; OP, oesophageal vessels; PO, peri-oesophageal; GP, gut plexus; DRV, dorsal ring vessel; LV, lateral vessel; VRV, ventral ring vessel; ST, stomach; VV, ventral vessel; SNV, supraneural vessel; ENV, efferent nephridial vessel; ANV, afferent nephridial vessel; MV, mucus vessel; EB, efferent branchial.

TABLE II

Estimated thicknesses of body, gill and blood vessel walls in *T. haplochaeta*.

Tissue	Thickness (mm)
Body dorsal wall	4.5×10^{-2} to 1.0×10^{-1}
Body ventral wall	8.5×10^{-2} to 1.0×10^{-1}
Gill wall	8.3×10^{-3} to 1.14×10^{-2}
Gill and body blood vessel wall	7.1×10^{-5} to 1.42×10^{-3}

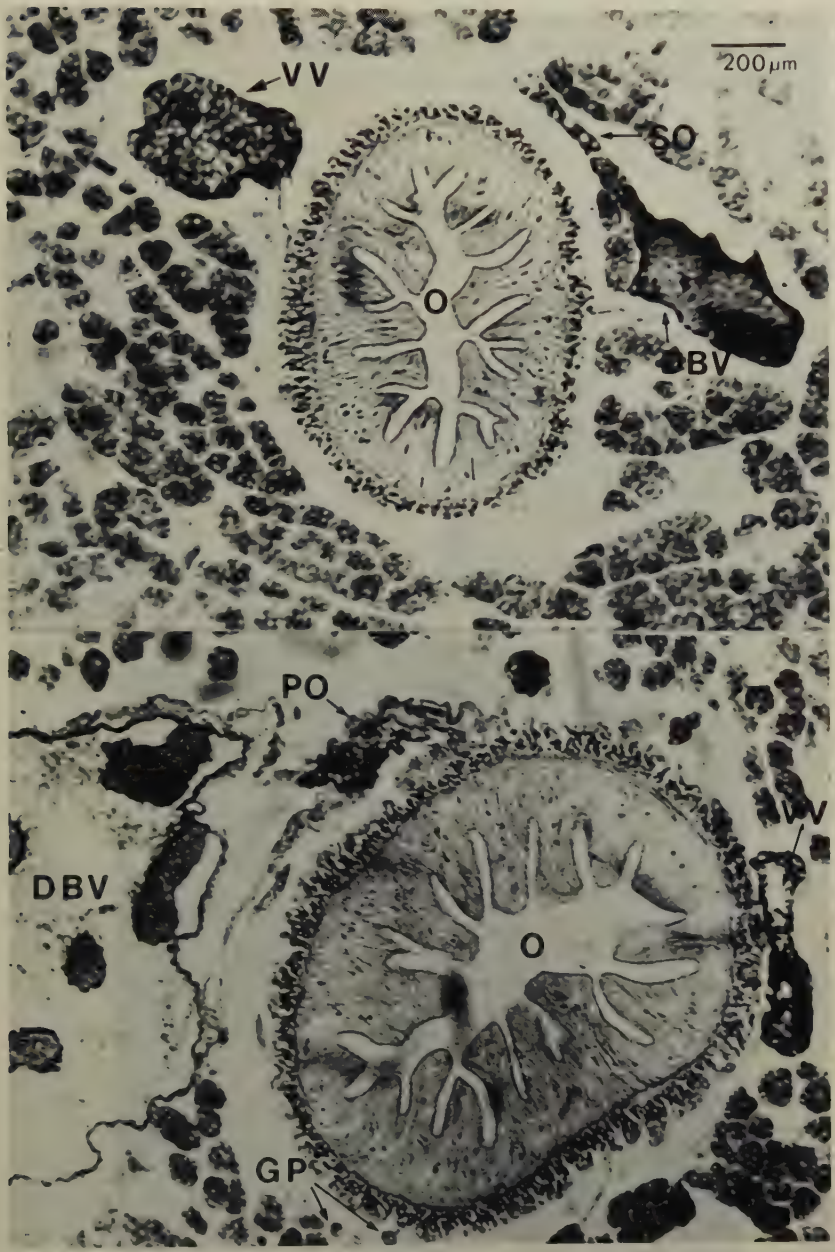


Fig. 8. Sections through the oesophageal regions of *T. haplochaeta* at the junction of the supra-oesophageal and dorsal vessels (above) and through the oesophageal ring vessel (below). O, oesophagus; DBV, dorsal vessel; PO, peri-oesophageal ring; GP, gut plexus; VV, ventral vessel; SO, supra-oesophageal vessel.

TABLE III

Estimated volumes and surface areas of body and gill of *T. haplochaeta*.

	Volume (V)	Surface area (SA)	SA : V
Body	1.62 cm^3	8.79 cm^2	5.4 : 1
Gill	3.32 mm^3	16.58 mm^2	5.0 : 1

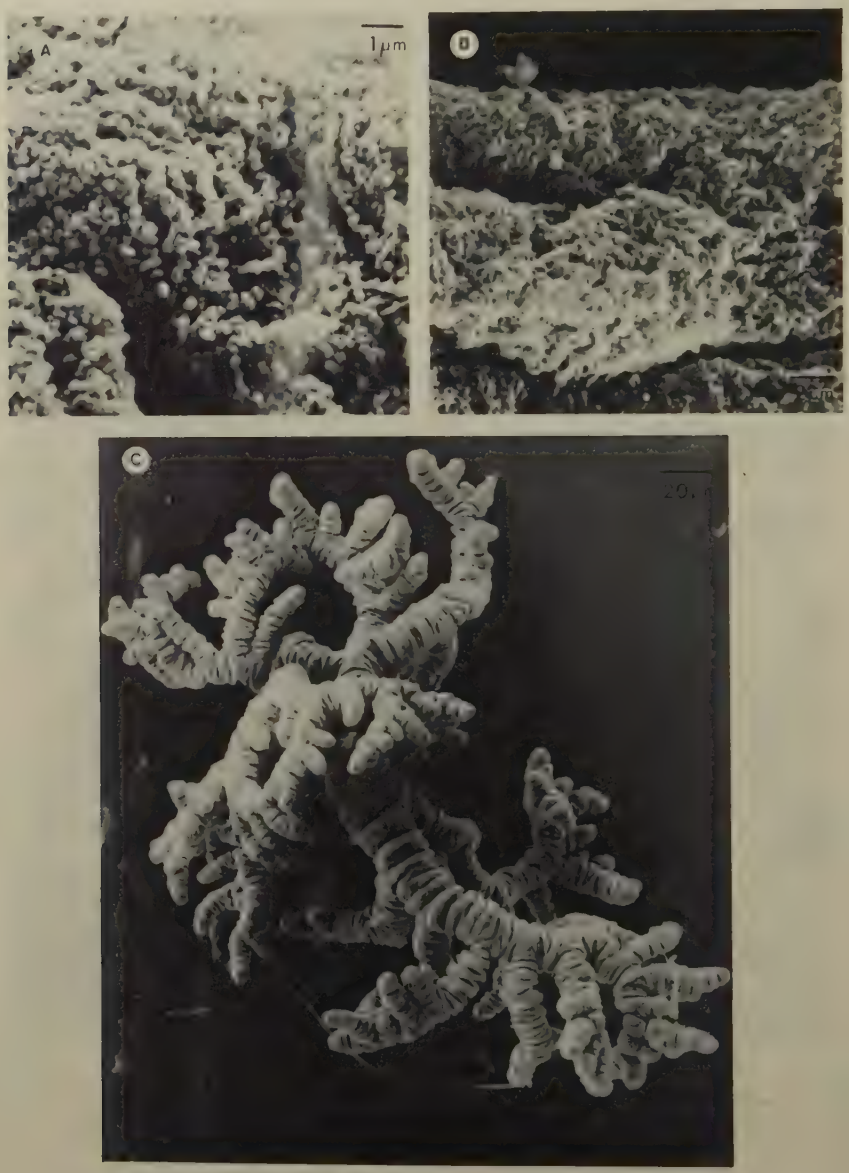


Fig. 9. Scanning electron micrograph of gill microsurface from *T. haplochaeta* at three magnifications showing the folded and microvillous appearance.

seems reasonable to assume that the thickness of the gill wall would be less than other parts of the body. This is shown in Table II where it may be seen that the gill wall is only one tenth the thickness of the ventral body wall. The wall thickness of blood vessels in the gills and body musculature was found to be identical but thinner than the gill wall itself.

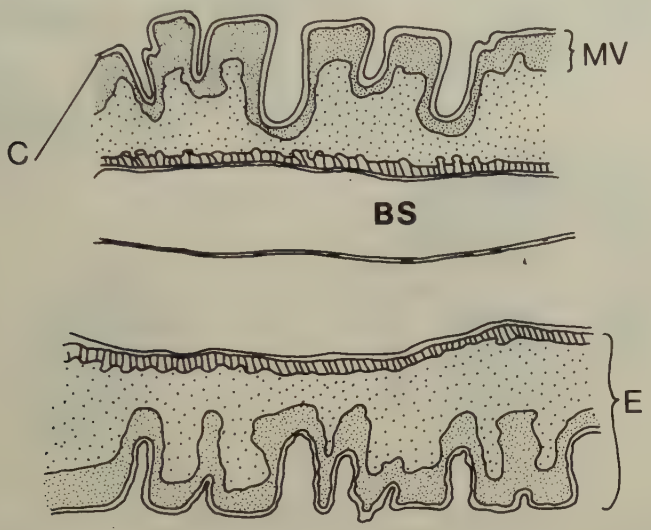


Fig. 10. Diagram of gill sectioned longitudinally showing the arrangement of blood spaces (BS) in relation to the cuticle (C), epithelium (E), and microvillous border (MV): ≈ ×45.

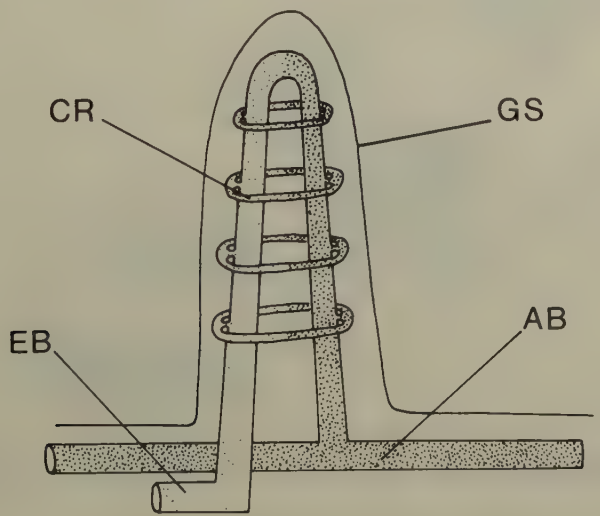


Fig. 11. Diagrammatic representation of the blood vessels and capillaries in a gill of *T. haplochaeta* showing the afferent branchial (AB), efferent branchial (EB), capillary ring (CR), and gill surface (GS): ≈ ×300.

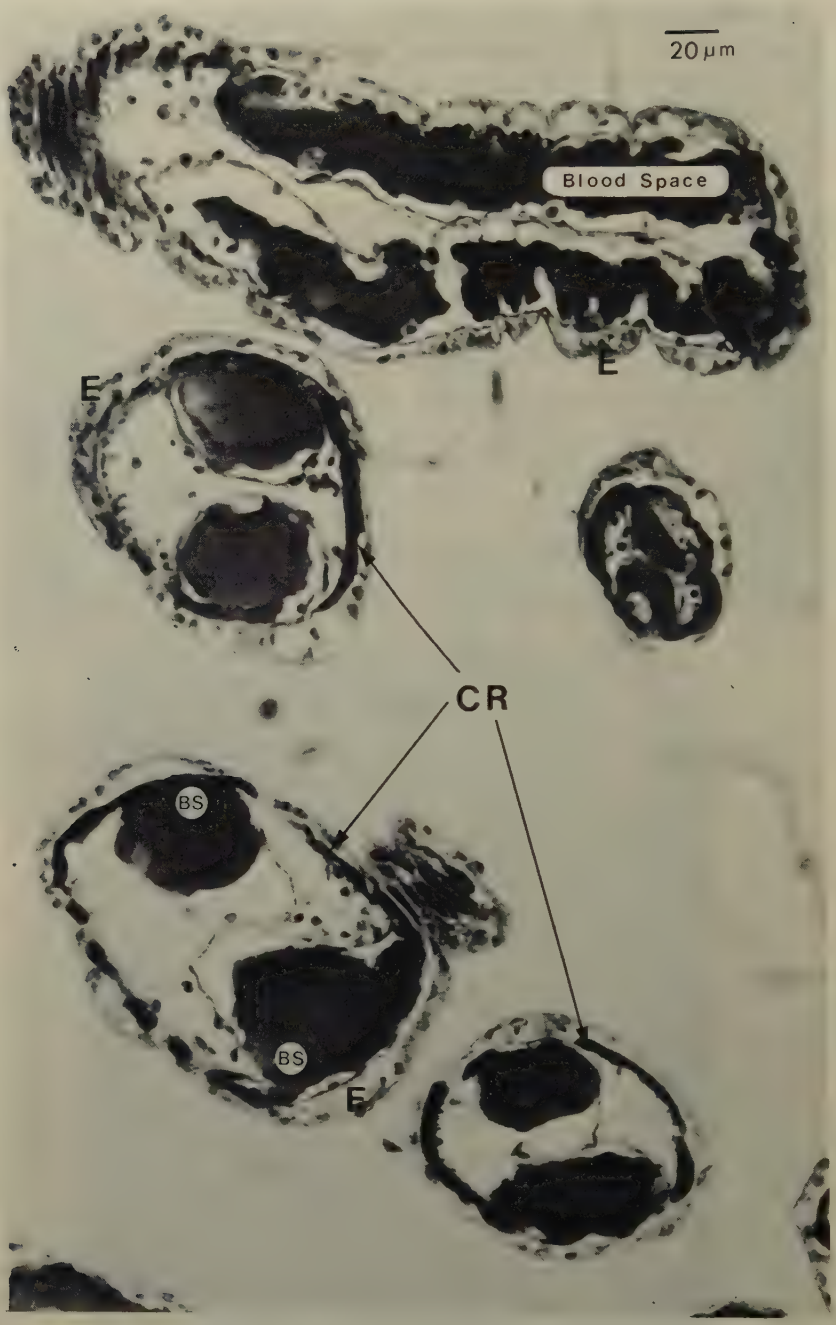


Fig. 12. Section through the gills of *T. haplochaeta* showing the arrangement of blood vessels within the gill branchlets: BS, blood space; CR, capillary ring; E, epithelium.

Estimated volume to surface area ratios obtained from the body and gills of *T. haplochaeta* are given in Table III. The body has the greater surface area to volume ratio and might appear to be a principal site for respiratory exchange. This assumption is, however, based purely on measurements made with the aid of a light microscope. Scanning electron micrographs of the gill surface (Fig. 9) reveal a much convoluted and greatly increased area than was previously observed and thus the contribution of the gill as an exchange surface may be greatly underestimated.

There is no coelomic space within the gill and blood spaces lie immediately adjacent to the epithelial layer (Fig. 10) so that the blood vessels within the gill of *T. haplochaeta* are applied closely to the epidermis. The blood vessels are arranged in the form of a loop, which ends at the tip of each branchia; thus there is almost certainly some mixing of afferent and efferent blood. The afferent and efferent vessels within the branchiae are connected by capillary rings which lie perpendicularly to the axes of the afferent and efferent vessels (Figs. 11 & 12). This arrangement closely resembles that found in the flabelligerid polychaete *Flabelliderma commensalis* (Spies, 1973) although the circulating fluid in the latter contains chlorocruorin rather than haemoglobin.

OXYGEN CONSUMPTION

Fig. 13 shows the relationship between dry tissue weight and oxygen uptake in *T. haplochaeta*. The data were fitted to the equation $Y = aX^b$, where Y is oxygen uptake in ml · h^{-1}, X is dry wt in g, b is a slope of the plot of log oxygen uptake against log dry weight and a is a proportionality factor.

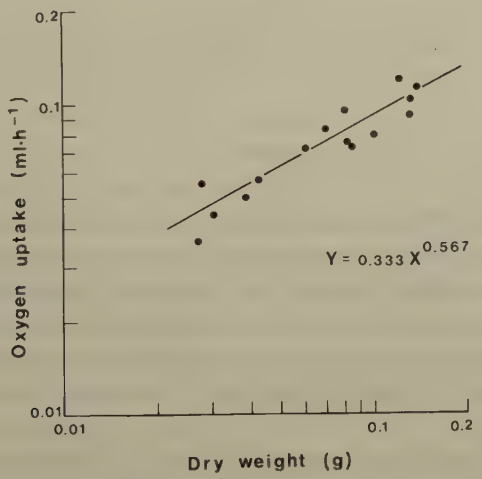


Fig. 13. Relationship between body size and oxygen uptake in *T. haplochaeta* at 20 °C: correlation coefficient r, fitted by least squares = 0.919.

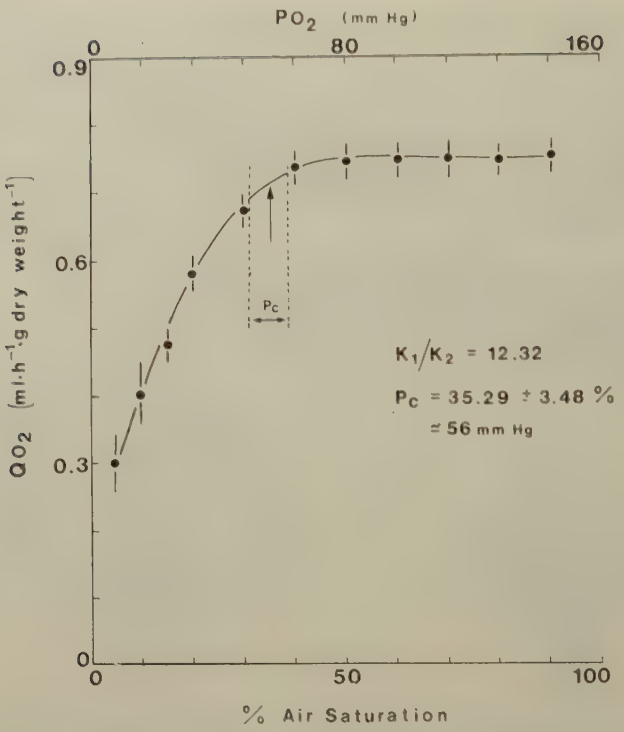


Fig. 14. Response of *T. haplochaeta* to declining oxygen tensions at 20 °C showing a zone of critical pressure (P_c) and the term K_1 K_2 quantifying the degree of oxyregulation (see text for further explanation): results plotted as mean ± SD for 10 worms of ≈0.1 g dry wt.

Fig. 14 shows the effect of declining oxygen tension on oxygen uptake in *T. haplochaeta*. It may be seen that between ≈35 and 100% air saturation the worms are oxygen-independent and that below 35% the animals become oxygen-dependent.

Discussion

When covered by the tide, *T. haplochaeta* exhibits a headward ventilatory stream similar to that seen in another terebellid, *Amphitrite ornata* (Mangum *et al.*, 1975), where peristaltic waves arise at the tail and move in a headward direction resulting in ventilation of the general body surface and gills. Thus the body surface receives oxygen at a high incurrent pO_2 and extracts some of this oxygen, allowing the anteriorly disposed gills to take oxygen from a lower tension in the burrow microenvironment.

Irrigatory behaviour has been shown to be influenced by the oxygen content of the water in several other species of polychaetes. Lindroth (1938) demonstrated that in *Neanthes* (= *Nereis*) *virens* irrigation increased with declining oxygen tensions

and was virtually continuous below ≈30% saturation. With a further decline in oxygen tension a point must eventually be reached at which the oxygen requirements for the energetic cost of irrigation itself are no longer met and there are then two possibilities for the animal: (i) activity may continue, the balance in demand for energy being made up by fermentation of glycogen or (ii) activity may cease, the worm becoming quiescent, so that the demand for oxygen is decreased. In *Neanthes* the rate of oxygen uptake is maintained down to the oxygen tension at which irrigation becomes continuous and below this point the rate of uptake declines along with activity. A similar response is seen in *Arenicola marina* (Wells, 1966) and since both species are intertidal, their respiratory behaviour may be adaptations to intermittent exposure. In addition, *Neanthes* and *Arenicola* may resort to aerial respiration by trapping bubbles of air within the burrows (Dales, 1969). Contrasting situations are seen in glycerid bloodworms which cease both ventilatory and circulatory movements under hypoxic conditions (Hoffman & Mangum, 1970), and in *Chaetopterus variopedatus* which becomes more active when the pO_2 falls below saturation and generates a vigorous ventilatory stream by the action of its broad, paddle-shaped gills (Dales, 1969). *Chaetopterus* is sublittoral and irrigation is prevented only if there is a burrow blockage.

Coyer & Mangum (1973) have shown that the frequency, duration, and amplitude of bursts of ventilation in the terebellid *Amphitrite ornata* change little with oxygen depletion. These ventilatory responses to reduced oxygen do not show close correlations within any particular polychaete family but are more likely to be related to differences in the animals' near environment.

An increase in the transport capacity of the blood in summer is consistent with the increased demand for oxygen at elevated acclimation temperatures which has been noted for a number of polychaetes (Mangum, 1978).

Although the capacity of the blood is high, the contents of the vessels occupy only 9% of the total body volume and are therefore unlikely to serve as a significant store of oxygen which might sustain the worm's respiration during phases of low tide. Unlike other terebellids (cf. Manwell, 1960; Garlick & Terwilliger, 1974; Terwilliger, 1974; Mangum *et al.*, 1975; Wells & Dales, 1975; Weber *et al.*, 1977), *Terebella haplochaeta* has no haemoglobin in its coelomic cells, the coelom occupying ≈28% of the body volume. Thus the coelomic store of oxygen cannot exceed the amount dissolved in physical solution. In this respect, our species closely resembles *Arenicola marina* for which Toulmond (1973) has documented variations in blood pO_2, pCO_2, pH, and oxygen saturation over the tidal cycle. He proposed that the vascular haemoglobin augments tissue oxygenation only during flood tide and is unable to function at low tide when the worm is abruptly cut off from its ventilated stream of oxygenated water.

The sigmoidal nature of the curves allows for the release of a large volume of bound oxygen for a comparatively small drop in pO_2. These properties of the haemoglobin-oxygen equilibrium are characteristic of most haemoglobins which function

in marine organisms inhabiting water of low ambient pCO_2 (reviewed by Mangum, 1976a; Weber, 1978b). Conversely, sigmoidal curves are unsuited for transport under declining oxygen tensions because oxygen is released over too narrow a range of pO_2. In addition, a Bohr effect confers no advantage while ambient carbon dioxide tensions are high. These conditions are likely to occur at low tide when oxygen transport is favoured by a hyperbolic curve and the absence of a Bohr effect (Mangum, 1976a; Weber, 1978b).

While determination of haemoglobin-oxygen equilibrium curves using carbon dioxide is undoubtedly a more accurate reflection of in ivo conditions than determinations in the presence of fixed acids, the influence of molecular carbon dioxide on the equilibrium (the so-called "carbamate" effect) cannot be distinguished from the Bohr effect which arises from the hydration reaction of carbon dioxide and results in a release of hydrogen ions. Although the carbamate effect has not been quantified in the present study, the extent of the Bohr shift was estimated using CO_2-free solutions of haemoglobin buffered to various pH values. Oxygen affinities were estimated by $P50$, which is the pO_2 at which half of the pigment is oxygenated. At pH 7.1 and 7.8 in 0.05 M Hepes buffers at 20 °C, the $P50$ decreased from 6.8 to 4.3 mm Hg. The Bohr coefficient ϕ, $= \Delta \log P50/\Delta pH$ was -0.28. We do not know the pH of the prebranchial blood in *T. haplochaeta* because the small size of these worms precludes such measurements. A pH range of 7.29–7.45 was, however, reported for prebranchial blood sampled from a variety of terebellids (Wells, 1974) and may be assumed to represent at least part of the physiological range.

The effect of pH on the shape of the equilibrium curve is similar to observations reported for the haemoglobins of several marine invertebrates (Weber, 1970, 1975, 1978b; Mangum, 1976a) but the interpretation of these findings does not suggest any clear adaptive significance. It is possible that the transition to a hyperbolic curve at low pH might conserve oxygen at low tide when carbon dioxide and the acidic end-products of glycolysis cannot easily be eliminated.

A low temperature sensitivity of the oxygen-binding reaction in *T. haplochaeta* (-21.6 kJ · mol^{-1}) may be considered as a homeostatic mechanism which secures stability in oxygen delivery over short term temperature fluctuations which might accompany a tidal cycle. It is interesting that a similar value of -28.5 was reported from the intertidal terebellid lugworms *Arenicola marina* (Weber, 1972) and *Abarenicola assimilis* (Chung & Ellerton, 1979). Greater temperature sensitivity has been shown for haemoglobins from the terebellids *Thelepus crispus* (Garlick & Terwilliger, 1974), *Terebella lapidaria* (Wells & Dales, 1975), and some arenicolids (Weber, 1972). Unfortunately it has not been possible to correlate these findings with temperature fluctuations occurring in the micro-environments inhabited by these organisms. It might be predicted that for intertidal invertebrates which are regularly exposed to sharp oscillations in temperature corresponding with the phase of the tide, the haemoglobin-equilibrium may be relatively insensitive to temperature. It is not immediately clear what benefit, if any, is conferred on the worm as the equilibrium

tends to a hyperbola at high temperatures. As with the pH effect on n, however, a hyperbolic curve may help to conserve oxygen when temperatures rise following exposure by the receding tide.

The enzyme carbonic anhydrase is found in various invertebrate tissues where it catalyses the reversible hydration of carbon dioxide (Maren, 1967). The presence of this enzyme is an important factor in determining whether carbon dioxide can be released with sufficient rapidity during the passage of blood through the tissues. The presence of carbonic anhydrase in the blood of the marine polychaetes *Arenicola marina* and *Terebella lapidaria* apparently aids the removal of metabolic carbon dioxide (Wells, 1973; Wells & Dales, 1975) and a similar rôle is postulated in *T. haplochaeta*.

The vascular system in annelids consists basically of a series of segmental vessels joining two longitudinal vessels, running from one end of the body to the other; one in the mid-dorsal line which flows forward and one in the corresponding ventral position in which the blood flows in the opposite direction. The ventral vessel lies just below the gut and dorsal to the nerve cord, beneath which a smaller (subneural) vessel is sometimes found. The ventral vessel gives rise to capillaries which surround the gut (Dales, 1967). The vascular system of *T. haplochaeta* resembles the vascular system of the terebellid *Amphitrite johnstoni* (= *Neoamphitrite figulus*) where the dorsal vessel seems to have disappeared in the region of the stomach and intestine and is functionally replaced by the lateral or paired ventral or gastric vessels (Dales, 1967). These unite into an enormous dorsal vessel giving rise to afferent branchials anteriorly. Until recently, ultrastructural studies of polychaete gills were lacking. Storch & Alberti (1978) have shown that the polychaete branchiae are true respiratory organs and they differ in this respect from animals such as the fishes and crustaceans where these organs perform both respiratory and ion absorption functions (Welsch & Storch, 1976). According to these studies there is good reason to believe that in different species of polychaetes the branchiae differ in their importance for gas exchange. Thus in the spionid *Malacoceros fuliginosus*, the afferent and efferent blood vessels are closed and there is no mixing of the separate cores of flow.

In other cases, the coelomic space does not project into the gills, thus permitting the blood vessels to come into closer contact with the epidermis (Orrhage, 1964; Siewing, 1976). A further reduction of the diffusion barrier between blood and water occurs in the branchiae of the nereid *Dendronereides heteropoda* (Gravier, 1934) and, on the basis of ultrastructural studies, in the gills of *Terebellides stroemi* (Storch & Alberti, 1978). These cases bear great similarity to the arrangement of blood spaces in the gill of *Terebella haplochaeta*.

Dales (1961) has studied oxygen uptake in three species of terebellids (*Eupolymnia heterobranchia*, *Neoamphitrite robusta*, and *Thelepus crispus*) and reported "b" values ranging from 0.43–0.74. Thus the "b" value reported here of 0.567 is well within this range and is also within the range reported for a variety of other polychaetes (Shumway, 1979).

The proportionality factor (0.333 for *Terebella haplochaeta*) may be used to compare the level of oxygen consumption between species. Shumway (1979) compared the "*a*" values of 13 different species of polychaetes and found that the worms fell into two groups with respect to oxygen uptake, errant polychaetes having a respiratory rate ≈ 2.4 times higher than sedentary ones. These groupings were based on readings taken at, or corrected to, 10 °C assuming a Q_{10} of 2.5. Using this same Q_{10} to correct the value given here for *T. haplochaeta* gives a reading of 0.133 and this value is similar to those reported for other sedentary polychaetes (Shumway, 1979) and in keeping with the tube-dwelling life style of *T. haplochaeta*.

Shumway (1979) reported eight species of polychaetes to be almost perfect oxyconformers. Mangum (1970, *Glycera dibranchiata*), May (1972, *Abarenicola pacifica*), Ewer & Fox (1940, *Sabella pavonina*) have all reported oxygen dependence for polychaetes in declining oxygen tensions. At least partial independence has been shown in the polychaetes *Schizobranchia insignis* (Dales, 1961), *Hyalinoecia tubicola* (Dales *et al.*, 1970), and *Nereis virens* (Hyman, 1932). All of these animals were categorized as oxygen dependent or independent by visual inspection of the graphed data.

Bayne (1971) proposed the use of an oxygen dependence index, K_1/K_2, to express an animal's capability to regulate its rate of oxygen uptake during exposure to declining oxygen tensions. This index is derived from the linear form of the equation for a hyperbola:

$$Q_{O_2} = pO_2/(K_1 + K_2 pO_2),$$

where Q_{O_2} is the weight-specific oxygen uptake in $ml \cdot g^{-1} \cdot h^{-1}$, pO_2 is the oxygen tension in mm Hg, and K_1 and K_2 are the intercept and slope, respectively, of a plot of pO_2 against pO_2/Q_{O_2}. The values of K_1 and K_2 which give the best fit through the points in Fig. 14 define the degree of oxyregulation by the ratio K_1/K_2. As the ratio K_1/K_2 increases, the rate of oxygen consumption becomes more directly proportional to oxygen tension (oxygen dependence) and as the ratio decreases, the rate of oxygen consumption approaches a constant (oxygen independence).

TABLE IV

Calculated K_1/K_2 values for eight species of polychaetes corrected for an animal of 0.1 g dry wt: data taken from Shumway (1979); *N* refers to the number of observations; for further explanation see text.

Species	K_1/K_2	*N*
Abarenicola affinis	119.9	12
Aphrodite aculeata	112.3	9
Arenicola marina	111.8	5
Eulalia heterobranchia	186.7	8
Glycera americana	221.4	10
Nereis diversicolor	170.6	6
Nereis virens	191.3	11
Perenereis nuntia	190.3	8

The oxygen dependence index, K_1/K_2, for *Terebella haplochaeta* of ≈ 0.1 g dry wt was found to be 12.32 ($n = 10$). This is a relatively low value and indicates a high degree of oxygen independence. This index has not yet been calculated for any other polychaetes; however re-calculating the data from Shumway (1979) gives the values shown in Table IV for eight species, all of which show rather high values for K_1/K_2. Several workers have applied this method to data derived from bivalve molluscs (Bayne, 1971, 1973; Taylor & Brand, 1975) and have shown that there is a general trend for increasing oxygen dependence with decreasing likelihood of encountering low environmental oxygen tensions in the natural habitat. Thus, animals which regularly experience low oxygen tensions in their natural habitats show low values for K_1/K_2. *T. haplochaeta* does not fit the general pattern shown by other polychaetes in that it shows good capabilities of oxyregulation; it does, however, lend further support to Bayne's theory in that it has a low K_1/K_2 ratio and is known to encounter low pO_2 levels regularly in this natural environment.

Perhaps the most interesting point to emerge from the investigation of declining oxygen tension on oxygen uptake is the zone of critical pressure (P_c), or the point at which the animal ceases to be oxygen independent and becomes oxygen dependent. In *T. haplochaeta* this was found to be between 30 and 40% of air saturation (calculated mean from data on which Fig. 14 is based is $35.39 \pm 3.5\%$ saturation). It may be that P_c is detected only where there is an irrigatory possibility of Q_{O_2} regulation, and that when worms are deprived of their tubes during respiratory measurements, a wide range of oxygen dependence will result and obscure P_c. It has already been shown that at $\approx 30.6\%$ saturation the worms' haemoglobin is almost totally deoxygenated and that the number of ventilatory waves $\cdot$ min^{-1} has decreased. It appears then, that at oxygen saturation levels $>30\%$ the combination of increased ventilation rate and high affinity haemoglobin provides the animals with enough oxygen to maintain a constant rate of oxygen uptake, whereas in the region of 30–40% saturation when ventilatory movements have decreased the worms are no longer able to obtain the oxygen supply needed to maintain a normal rate of oxygen uptake.

Acknowledgements

The authors acknowledge useful discussions with Professor R.P. Dales and Dr. I.D. Marsden. The project was financed by an equipment grant from the Auckland University Research Committee.

References

Antonini, E. & M. Brunori, 1971. *Hemoglobin and myoglobin in their reaction with ligands.* North-Holland, Amsterdam, 436 pp.

Bayne, B.L., 1971. Oxygen consumption by three species of lamellibranch mollusc in declining ambient oxygen tension. *Comp. Biochem. Physiol.*, Vol. 40A, pp. 955–970.

BAYNE, B. L., 1973. The responses of three species of bivalve mollusc to declining oxygen tension at reduced salinity. *Comp. Biochem. Physiol.*, Vol. 45A, pp. 793–806.

CHUNG, M. C. M. & H. D. ELLERTON, 1979. The physico-chemical and functional properties of extracellular respiratory haemoglobins and chlorocruorins. *Prog. Biophys. molec. Biol.*, Vol. 35, pp. 53–102.

COYER, P. E. & C. P. MANGUM, 1973. Effect of temperature on active and resting metabolism in polychaetes. In, *Effects of temperature on ectothermic organisms, ecological implications and mechanisms of compensation*, edited by W. Wieser, Springer-Verlag, Heidelberg, pp. 173–180.

CRISP, M., J. DAVENPORT & S. E. SHUMWAY, 1978. Effects of feeding and of chemical stimulation on the oxygen uptake of *Nassarius reticulatus* (Gastropoda: Prosobranchia). *J. mar. biol. Ass. U.K.*, Vol. 58, pp. 387–399.

DACIE, J. V. & S. M. LEWIS, 1975. *Practical haematology*. Churchill Livingstone, Edinburgh, 5th edition, 629 pp.

DALES, R. P., 1961. Oxygen uptake and irrigation of the burrow by three terebellid polychaetes: *Eupolymnia*, *Thelepus*, and *Neoamphitrite*. *Physiol. Zoöl.*, Vol. 34, pp. 306–311.

DALES, R. P., 1967. *Annelids*. Hutchinson, London, 200 pp.

DALES, R. P., 1969. Respiration and energy metabolism in annelids. In, *Chemical zoology, Vol. 4*, edited by M. Florkin & B. J. Scheer. Academic Press, New York, pp. 93–109.

DALES, R. P., C. P. MANGUM & J. C. TICHY, 1970. Effects of changes in oxygen and carbon dioxide concentrations on ventilation rhythms in onuphid polychaetes. *J. mar. biol. Ass. U.K.*, Vol. 50, pp. 365–380.

EWER, R. F. & H. M. FOX, 1940. On the function of chlorocruorin. *Proc. R. Soc. Ser. B*, Vol. 129, pp. 137–153.

GARLICK, R. L. & R. C. TERWILLIGER, 1974. Coelomic cell hemoglobin of the terebellid polychaete, *Thelepus crispus* Johnson. Structure and oxygen equilibrium. *Comp. Biochem. Physiol.*, Vol. 47B, pp. 543–553.

GRAVIER, C., 1934. Remarques sur la morphologie du parapode des Néréidiens. *Annls Sci. nat.*, Vol. 17, pp. 347–367.

HILL, A. V., 1910. The possible effects of aggregation of haemoglobin on its dissociation curve. *J. Physiol., Lond.*, Vol. 40, pp. iv–vii.

HOFFMAN, R. J. & C. P. MANGUM, 1970. The function of coelomic cell hemoglobin in the polychaete *Glycera dibranchiata*. *Comp. Biochem. Physiol.*, Vol. 36, pp. 211–228.

HUMASON, G. L., 1972. *Animal tissue techniques*. W. H. Freeman & Co., San Francisco, 641 pp.

HYMAN, L., 1932. Relation of oxygen tension to oxygen consumption in *Nereis virens*. *J. exp. Zool.*, Vol. 61, pp. 209–221.

LINDROTH, A., 1938. Studien über die respiratorischen mechanismen von *Nereis virens* (Sars). *Zool. Bidr. Upps.*, Vol. 17, pp. 367–497.

MANGUM, C. P., 1970. Respiratory physiology in annelids. *Am. Sci.*, Vol. 58, pp. 641–647.

MANGUM, C. P., 1976a. Primitive respiratory adaptations. In, *Adaptation to environment*, edited by R. C. Newell, Butterworths, London, pp. 191–278.

MANGUM, C. P., 1976b. The function of respiratory pigments in estuarine animals. *Estuarine processes, Vol. 1, Uses, stresses, and adaptations to the estuary*, edited by M. Wiley, Academic Press, New York, pp. 356–380.

MANGUM, C. P., 1976c. The oxygenation of hemoglobin in lugworms. *Physiol. Zöol.*, Vol. 49, pp. 85–99.

MANGUM, C. P., 1978. Temperature adaptation. In, *Physiology of annelids*, edited by P. J. Mill, Academic Press, London, pp. 447–478.

MANGUM, C. P. & W. VAN WINKLE, 1973. Responses of aquatic invertebrates to declining oxygen conditions. *Am. Zool.*, Vol. 13, pp. 529–541.

MANGUM, C. P., B. R. WOODIN, C. BONAVENTURA, B. SULLIVAN & J. BONAVENTURA, 1975. The role of coelomic and vascular hemoglobin in the annelid family Terebellidae. *Comp. Biochem. Physiol.*, Vol. 51A, pp. 281–294.

MANWELL, C., 1960. Histological specificity of respiratory pigments. I. Comparisons of the coelom and muscle hemoglobins of the polychaete worm *Travisia pupa* and the echiuroid worm *Arynchite pugettensis*. *Comp. Biochem. Physiol.*, Vol. 1, pp. 267–276.

MAREN, T. H., 1967. Carbonic anhydrase: chemistry, physiology and inhibition. *Physiol. Rev.*, Vol. 47, pp. 595–781.

MAY, D. R., 1972. The effects of oxygen concentration and anoxia on respiration of *Abarenicola*

pacifica and *Lumbrinereis zonata* (Polychaeta). *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 142, pp. 71–83.

MENZIES, R. J., 1968. A small basin containing anoxic sediments. *Int. Rev. ges. Hydrobiol.*, Vol. 53, pp. 77–81.

NEWELL, R. C., 1973. Factors affecting the respiration of intertidal invertebrates. *Am. Zool.*, Vol. 13, pp. 513–528.

ORRHAGE, L., 1964. Anatomische und morphologische Studien über die Polychaetenfamilien Spionidae, Disomidae und Poecilochaetidae. *Zool. Bidr. Upps.*, Vol. 36, pp. 335–405.

ROSSI-FANELLI, A., E. ANTONINI & A. CAPUTO, 1964. Hemoglobin and myoglobin. *Adv. Protein Chem.*, Vol. 19, pp. 74–222.

SHUMWAY, S. E., 1979. The effects of body size, oxygen tension and mode of life on the oxygen uptake rates of polychaetes. *Comp. Biochem. Physiol.*, Vol. 64A, pp. 273–278.

SIEWING, R., 1976. Probleme und neuere Erkenntnisse in der Großsystematik der Wirbellosen. *Verh. dt. zool. Ges.*, Vol. 69, pp. 59–83.

SPIES, R. B., 1973. The blood system of the flabelligerid polychaete *Flabelliderma commensalis* (Moore). *J. Morph.*, Vol. 139, pp. 465–490.

STORCH, V. & G. ALBERTI, 1978. Ultrastructural observations on the gills of polychaetes. *Helgoländer wiss. Meeresunters.*, Vol. 31, pp. 169–179.

TAYLOR, A. C. & A. R. BRAND, 1975. The effects of hypoxia and body size on the oxygen consumption of the bivalve *Arctica islandica*. *J. exp. mar. Biol. Ecol.*, Vol. 19, pp. 187–196.

TERWILLIGER, R. C., 1974. Oxygen equilibria of the vascular and coelomic hemoglobins of the terebellid polychaete, *Pista pacifica*. Evidence for an oxygen transfer system. *Comp. Biochem. Physiol.*, Vol. 48A, pp. 745–755.

TOULMOND, A., 1973. Tide-related changes of blood respiratory variables in the lugworm *Arenicola marina* (L.). *Respir. Physiol.*, Vol. 19, pp. 130–144.

VENABLE, J. H. & R. COGGESHALL, 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.*, Vol. 25, pp. 407–413.

WEBER, R. E., 1970. Relations between functional and molecular properties of annelid haemoglobins. Interaction between haems in the haemoglobin of *Arenicola marina*. *Comp. Biochem. Physiol.*, Vol. 35, pp. 179–189.

WEBER, R. E., 1972. On the variation in oxygen-binding properties in haemoglobins of lugworms (Arenicolidae, Polychaeta). In, *Proc. 5th Europ. mar. Biol. Symp.*, edited by B. Battaglia, Piccin, Padova, pp. 231–243.

WEBER, R. E., 1975. Respiratory properties of haemoglobins from eunicid polychaetes. *J. Comp. Physiol.*, Vol. 99, pp. 297–307.

WEBER, R. E., 1978a. Respiration. In, *Physiology of annelids*, edited by P. J. Mill, Academic Press, London, pp. 369–392.

WEBER, R. E., 1978b. Respiratory pigments. In, *Physiology of annelids*, edited by P. J. Mill, Academic Press, London, pp. 393–446.

WEBER, R. E., C. P. MANGUM, H. STEINMAN, C. BONAVENTURA, B. SULLIVAN & J. BONAVENTURA, 1977. Hemoglobins of two terebellid polychaetes: *Enoplobranchus sanguineus* and *Amphitrite ornata*. *Comp. Biochem. Physiol.*, Vol. 56A, pp. 179–187.

WELLS, G. P., 1966. The lugworm (*Arenicola*) – a study in adaptation. *Neth. J. Sea Res.*, Vol. 3, pp. 294–313.

WELLS, R. M. G., 1973. Carbonic anhydrase activity in *Arenicola marina* (L.). *Comp. Biochem. Physiol.*, Vol. 46A, pp. 325–331.

WELLS, R. M. G., 1974. Hydrogen ion activity in polychaete body fluids. *Comp. Biochem. Physiol.*, Vol. 49A, pp. 567–574.

WELLS, R. M. G. & R. P. DALES, 1975. Haemoglobin function in *Terebella lapidaria* L., an intertidal terebellid polychaete. *J. mar. biol. Ass. U.K.*, Vol. 55, pp. 221–220.

WELLS, R. M. G. & R. P. DALES, 1976. Subunit organization in the respiratory proteins of the Polychaeta. *Comp. Biochem. Physiol.*, Vol. 54A, pp. 387–394.

WELSCH, U. & V. STORCH, 1976. *Comparative animal cytology and histology*. Sidgwick & Jackson, London, 343 pp.

J. exp. mar. Biol. Ecol., 1980, Vol. 46, pp. 279–296
© Elsevier/North-Holland Biomedical Press

INTERACTION OF MINERAL ELEMENTS IN SEA WATER AND SHELL OF OYSTERS (*CRASSOSTREA VIRGINICA* (Gmelin)) CULTURED IN CONTROLLED AND NATURAL SYSTEMS

MELBOURNE R. CARRIKER, ROBERT E. PALMER, LOWELL V. SICK and CONSTANCE C. JOHNSON

College of Marine Studies, University of Delaware, Lewes, DE 19958, U.S.A.

Abstract: The interaction is reported of selected chemical elements (cadmium, calcium, copper, iron, magnesium, manganese, strontium, and zinc) in cultured sea water, with soft tissues, prismatic calcite of the right valve, and foliated calcite of right and left valves of genetically similar American oysters, *Crassostrea virginica* (Gmelin) grown in a natural habitat and in two environmentally controlled experimental systems (flow-through and recycle). The addition of trace elements as algal nutrients in ambient sea water was reflected in higher concentrations of trace metals in shells and soft tissues of oysters grown in experimental systems. Calcium was relatively uniformly distributed in major regions of valves from the three habitats, even though its concentration fluctuated widely in sea water in experimental systems. Magnesium and strontium were most concentrated in valves of oysters grown in the recycle system (magnesium in the prismatic layer of the shell and strontium in the foliated calcite). Iron was uniformly distributed. Cadmium, copper, manganese, and zinc were most concentrated in the prismatic calcite of valves from the flow-through system. In soft tissues, calcium was more concentrated in oysters from experimental systems than in those from the natural habitat. Manganese was about equally distributed in soft tissues from the three habitats, whereas copper and iron were more concentrated in soft tissues in experimental systems than in the natural habitat, and were many times more concentrated in soft tissues than in valves from all three habitats. As concentrations of magnesium, strontium, mangenese, zinc, and cadmium increased in valves in experimental systems, pigmentation of valves decreased. The study confirmed the capacity of oysters to concentrate several elements in their valves as concentration of these elements increased in ambient sea water and disclosed the heterogeneous distribution of these elements in major regions of the valves.

INTRODUCTION

Active incorporation of different kinds and concentrations of minor and trace elements during molluscan shell formation is influenced by a number of factors (Dodd, 1967; Wilbur, 1972, 1976). These include taxonomic differences (Turekian & Armstrong, 1960), ontogenetic stages (Swan, 1956; Krasnov & Pozdnyakova, 1975), concentration of elements in ambient water and variations of these with seasonal and environmental changes (Odum, 1957; Dodd, 1965; Lerman, 1965; Pilkey & Harriss, 1966; Krasnov & Pozdnyakova, 1975; Frazier, 1976; Buchardt & Fritz, 1978), salinity (Rucker & Valentine, 1961; Pilkey & Goodell, 1963; Dodd, 1966), temperature (Rucker & Valentine, 1961; Pilkey & Goodell, 1963), physiological conditions of shell formation (Simkiss, 1976), mineralogy of the shell (ara-

gonitic or calcitic; Harriss, 1965; Wada & Suga, 1976), and variation in shell layers even within the same type of mineral (Dodd, 1967; Wada & Suga, 1976).

The chemical composition of shell of bivalves grown actively in controlled systems tends to reflect the concentrations of elements present in the culture water (Romeril, 1971, zinc; Sturesson, 1976, lead, 1978, cadmium; Lorens & Bender, 1977, magnesium; Conger *et al.*, 1978; Sick *et al.*, 1979, calcium). Whether this is true for all elements incorporated in shell, what natural minimal and maximal limits in concentration exist in shell for these and other elements, and how changes in concentration and kinds of elements affect shell morphology and ultrastructure are not known (Palmer & Carriker, 1979).

Calcium, the major cation in shell, probably influences the incorporation of other elements in shell, but to what degree this occurs and what elements are affected are matters which need investigation (Odum, 1957; Romeril, 1971). Trace and minor elements can be incorporated in shell as substitutes for calcium in the calcitic or aragonitic crystal lattice, as constituents of separate mineral phases and organic compounds, and absorbed on exterior surfaces (Dodd, 1967; Carriker, 1978; Carriker *et al.*, 1978).

Frazier (1975, 1976) investigated the seasonal dynamics of metals in the shell and soft tissues of hatchery-reared American oysters held in estuaries in Maryland, but the dynamics of several elements at a time in oysters growing in controlled experimental sea-water systems has not been examined.

This paper reports the results of a preliminary study of the interaction of mineral elements in sea water with the soft tissues and three major regions of the shell of *Crassostrea virginica* (Gmelin). Concentration and distribution of cadmium, calcium, copper, iron, magnesium, manganese, strontium, and zinc were emphasized. Hatchery-raised oysters were grown for 3 months in three different habitats: a natural area with excellent growing conditions (Broadkill River, Delaware), a controlled experimental environmental system in which sea water was changed every second day (a "flow-through" system), and a controlled experimental environmental system utilizing largely recycled filtered sea water ("recycle" system).

Materials and Methods

Oysters for the study developed from a single spawning on 26 May, 1977 of two oysters collected in the Broadkill River, Delaware. Larvae and spat were raised in the experimental maricultural facility of the College of Marine Studies, University of Delaware, Lewes, Delaware (see Palmer & Carriker, 1979, for details). After 17 days, pediveligers were allowed to set on large rectangular sheets of mylar film until spat density was $\approx 1/cm^2$. The mylar was cut into six pieces, each inserted into a Plexiglas frame $\approx 20 \times 30$ cm, and spat were held in large fiberglass tanks at 27 °C (sea water was changed every second day) with a surplus of food for another

17 days. Spat, approximately 5 mm in height, were then thinned to about 100 on each mylar sheet. Two frames were positioned in each of the three environments: Broadkill River, the maricultural experimental flow-through system, and the maricultural experimental recycle system, where they remained until termination of the experiment on 17 September, 1977.

DESCRIPTION OF HABITATS

Broadkill River

This is a tidal estuary which drains a large part of *Spartina alterniflora*-dominated Great Marsh, Lewes, Delaware. Two oyster frames were suspended from a floating dock in the Broadkill River at a point ≈0.25 mile before it flows through Roosevelt Inlet into Delaware Bay. The estuary is characterized by swift tidal currents, muddy bottom, and extremely high levels of turbidity and organic matter. Oyster frames were suspended ≈1 m off the bottom at low tide and oriented horizontally so that the surface of the mylar to which oysters were attached faced downward. This prevented burial of the oysters by silt. Frames were removed from the water five times during the 11-wk period to rid them and the oysters of fouling organisms, chiefly the tunicate, *Molgula manhattensis*.

Experimental systems

The flow-through and recycle systems were similar in physical configuration, but differed significantly in treatment and frequency of change of the water. In both systems, oyster frames were positioned in shallow fiberglass tanks, and, as with the Broadkill oysters, oriented so that the mylar attachment surface faced downward. Tanks were approximately 200 × 40 × 15 cm in size and held ≈500 l each. Oysters in both systems were fed once or twice a day on an algal mixture of *Thalassiosira pseudonana* and *Isochrysis galbana*. Oysters in the flow-through system were fed an average daily ration of 1.92×10^8 cells/g wet wt; those in the recycle system were fed 6.66×10^7 cells/g wet wt. In both systems oysters were removed from the water once daily and rinsed briefly with fresh water. Exposure of oysters to the air was normally for <1.5 h, although on 10 of 78 days it extended for 5.5 to 7 h.

All of the water in the flow-through experimental system was replaced every 2 days. New water for this system came from a mixture of Indian River Inlet sea water and Lewes city tap water, and was mixed in the oyster tank to an approximate salinity of 20‰. Indian River sea water, ≈30–32‰ *S*, was passed through one 5-μm and two 1-μm cartridge filters before being pumped into the oyster tank.

In the recycle experimental system, ≈90–95% of the water, also 20‰ *S*, was reused daily (Pruder & Bolton, 1977). The only major addition of new water to this system was 35 l/day of *I. galbana* suspension, cultured in a mixture of Indian River sea water and Lewes tap water. *Thalassiosira pseudonana* was cultured in water from

the recycle oyster tanks; after that water was passed daily through cartridge filters, a foam fractionator, and a charcoal filter.

Oysters in both experimental systems were exposed to continuous fluorescent illumination, which was slightly more intense in the recycle than in the flow-through experimental system. In addition, calcium carbonate was added ad lib. in both systems in an attempt to maintain constant concentrations of dissolved calcium.

The three oyster-growing environments thus included a productive natural estuary, a culture system utilizing diluted clean sea water changed every second day, and a second culture system utilizing largely recycled, filtered, and diluted sea water. These habitats provided widely different environmental conditions in which to examine the interaction of mineral elements between oysters and the ambient media.

Environmental factors

In the Broadkill River, data on temperature, salinity, and concentration of dissolved and particulate organic matter were collected at hourly intervals over a full tidal cycle on three occasions during the 79 days (2 July–17 September) oysters were in the river (Palmer & Carriker, 1979) (Table I).

In each of the experimental systems, data were available daily for temperature, pH, and number of cells of *Isochrysis galbana* and *Thalassiosira pseudonana* provided as food/g of oyster (Epifanio, 1976). Data recorded on a weekly basis included salinity and calcium concentration in the ambient water.

Oyster valves

A hole was made by removing a fifth of one side of the right valve of each oyster with a thin, polished, high speed, diamond wheel (22 mm diameter) under running tap water. Adductor muscles were cut from the valves with a plastic knife inserted through the hole, and soft tissues were set aside for freezing and subsequent chemical analyses. Shells were immersed in 20% clorox solution (1% sodium hypochlorite) for 10 s. in standing tap water for 10 s, and in running tap water for 10 s. Then they were blotted dry on paper towelling, dried in an oven at 65 °C for 15 min, and stored in plastic dishes in a cabinet at room temperature. Valves from the maricultural systems were fouled only with algae which was removed by the clorox treatment. Valves from the Broadkill River were heavily fouled with naturally setting oyster spat, various other sessile organisms, and detritus. These were scraped off, and the exterior surfaces of the valves were scrubbed with a glassware brush. Nine months after the initial processing of the valves, four sets of valves from the Broadkill River and five each from the two experimental systems were selected and prepared for chemical analysis in an ultraclean laboratory having positive pressure, laminar flow and filtered air. Valves from each oyster, treated individually, were soaked in full strength clorox for several minutes, rinsed several times in quartz distilled water, and drained dry. Prismatic shell from the right valve (broken off with plastic

forceps), the remaining foliated calcite of the right valve, and the entire left valve (predominantly foliated calcite) of each oyster were then ground to a fine powder in a glass mortar and pestle. Each of the three major regions of each pair of valves was stored separately for quantitative analysis of the elements cadmium, calcium, copper, iron, magnesium, manganese, strontium and zinc by atomic absorption spectrophotometry. For increased accuracy, where possible, approximately the same weight of shell sample was used for each determination. Sufficient mass of prismatic shell was not always available, and inconsistent results obtained with small weights (<20 mg) were discounted. Samples, a total of 84 shell fractions and soft tissues, were digested by low-temperature oxygen plasma ashing for 36 h. Volatilization of the more unstable elements (e.g., cadmium) was prevented by using a power setting for the ashing process of 100 W. Ashed samples were dissolved in 70% double quartz-distilled nitric acid, placed in the Teflon liner of a Parr 25-ml bomb, sealed, and further digested at 90 °C for 2 h. Following wet digestion, samples were filtered through 0.45-μm nucleopore membrane filters (Lot No. 4569) and brought to volume with double quartz distilled 6% nitric acid. Samples were analyzed with a Perkin–Elmer atomic absorption spectrophotometer by carbon rod atomization, except calcium, which was measured using flame atomization. The instrument was housed within a clean laboratory complex and featured simultaneous background deuterium arch correction, electrodeless discharge power supply and lamp, automatic sample injection system, and computer phone patch coupling. Quality control consisted of using National Bureau of Standards certified bovine liver at freeze drying, ashing, and filtration steps in the laboratory procedure.

Although trace metal concentrations were not synoptically monitored in the experimental systems during the course of the growth experiments, a 3-month survey was initiated toward the end of the growth experiment (i.e., September, 1977; see Table II) and was felt to be representative of typical trace metal concentrations in the systems. In addition, sampling among the various components of the culture systems provided a relative index of trace metal partitioning within the recirculating and flow-through systems.

Water samples analyzed for dissolved trace metals were collected in selected components of the experimental systems (i.e., a combination of water from Indian River Inlet and local fresh water, water from algal and oyster growing tanks, and the dissolved fraction of the foam fractionator and in the Broadkill River). Samples were immediately filtered through 0.45-μm nucleopore membrane filters, acidified to a pH of 2, and frozen until analyzed. Before analysis, water samples were treated by solvent extraction techniques, using methyl isobutyl ketone, ammonium pyrrolidine dithocarbamate, and diethylammonium diethyldithiocarbamate (Kinradi & Van Loon, 1974). All dissolved trace metals were analyzed by carbon rod atomization coupled with atomic absorption spectrophotometry, except calcium, which was measured by flame atomization.

RESULTS

Environmental conditions

Mean values and ranges for temperature, salinity, and pH of the water in the three habitats in which oysters were grown are given in Table I. The two experimental systems differed from the Broadkill River in having lower, more constant temperatures and lower salinities. Temperature, salinity, and pH were similar in the two experimental systems. Although pH was not monitored in the Broadkill River,

TABLE I

Average values for environmental variables measured in each of the three oyster growing habitats during the period 1 July 17 September, 1977: ranges are shown in parentheses; averages are based on monthly samples in the Broadkill River and daily observations in each of the experimental maricultural systems.

Variable	Broadkill	Flow-through	Recycle
Temperature (°C)	23.4 (19.3–30.1)	19.2 (17.1–21.5)	19.5 (17.5–21.4)
Salinity (‰)	29.3 (24.0–32.0)	20.0 (20.0–20.5)	19.0 (17.0–19.5)
pH		7.94 (7.69–8.27)	7.88 (7.71–8.08)

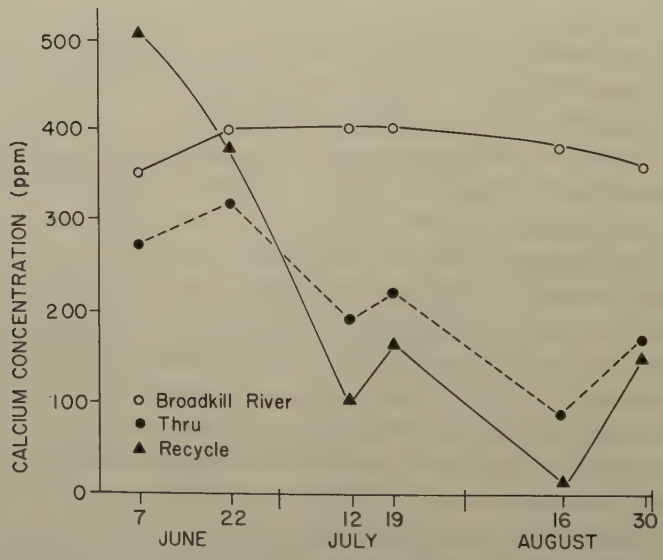


Fig. 1. Average monthly calcium concentrations in Broadkill River, experimental flow-through and recycle systems, for the period June–September, 1977: values are means based on five replicate determinations per sample; SD in all cases was <6%.

monitoring done in the river in connection with other studies indicated that pH probably remained above 8 during this time of the year (Palmer & Carriker, 1979). Particulate organic carbon and dissolved organic carbon averaged 3.06 and 3.96 mg/l, respectively, in the Broadkill River during the summer of 1977.

Calcium concentrations ranged from about 300–400 ppm in the Broadkill River during the experimental period of July–September. In the two experimental systems, calcium concentration fluctuated widely, between ≈ 20 and 500 ppm (Fig. 1). Low calcium concentrations were particularly pronounced in the recycle system where concentrations were below 150 ppm during 5 of 11 wk of culture.

Morphology and growth of oyster valves

At the termination of the experiment, shape and appearance of valves of oysters (114 days old) grown in the three habitats were similar. There were no obvious deformities or anomalies (Galtsoff, 1969). Shells of all oysters were substantial and solid, but those of recycle oysters, which contained almost no chalky deposits, were thicker than flow-through valves. Recycle valves were denser than both flow-through and Broadkill valves. Oysters grown in the Broadkill River attained a considerably larger size (mean height 47.4 ± 4.9 mm) than flow-through oysters (21.2 ± 1.6 mm) and recycle oysters (21.0 ± 1.4 mm) (Palmer & Carriker, 1979).

Trace elements in experimental systems

Analyses of samples collected simultaneously from several components of the experimental systems indicated that highest concentrations of trace metals were associated with phytoplankton culture tanks (Table II). In the recycling culture system, dissolved iron and zinc, for example, were one and two orders of magnitude higher, respectively, in the phytoplankton culture tanks than in the oyster culture tanks. For all five trace metals analyzed, significantly higher concentrations ($P < 0.05$) were found in phytoplankton cells than in soft oyster tissues. Highest concentrations of both dissolved and cell trace metals were also associated with the phytoplankton culture tank in the flow-through system.

In general, highest concentrations of trace metals were found in the flow-through culture system, relative to comparable samples measured in the recycle system (Table II). For example, concentrations of dissolved iron in the phytoplankton growing tanks of the flow-through system were 18.1 ppb compared to 8 ppb for the phytoplankton tank of the recycle system. Similarly, phytoplankton cells collected from the flow-through system had an iron concentration of 1205 ppm relative to an average concentration of 658 ppm in cells grown in the recycle system. Although copper was the only trace metal analyzed that did not have a higher dissolved concentration in the flow-through system relative to the recycle system, higher tissue concentrations of copper were found in both phytoplankton and soft tissues of oysters grown in the flow-through system.

TABLE II

Concentrations of selected trace metals in the abiotic and biotic components of the experimental culture systems and the Broadkill River: samples in the Broadkill were collected during the growth experiment, while samples in the experimental culture systems were collected between September and December, 1977; values are averages and standard errors based on three (experimental systems) and four (Broadkill River) monthly replicate samples and five determinations per sample.

Habitat and fractions	Trace metal				
	Cd	Cu	Fe	Mn	Zn
Experimental: recycle					
Incoming sea water (ppb)	0.28 ± 0.02	9.00 ± 1.10	20.0 ± 3.41	0.93 ± 0.07	31.0 ± 5.90
Fresh water (ppb)	0.09 ± 0.07	7.20 ± 0.80	82.0 ± 5.65	0.33 ± 0.01	42.0 ± 6.10
Nutrient mix (ppm)	<0.0009	0.90 ± 0.07	234.0 ± 12.20	18.0 ± 0.99	1.8 ± 0.91
Algal growth tank					
Dissolved (ppb)	1.49 ± 0.11	6.28 ± 0.41	8.0 ± 0.96	1.06 ± 0.09	50.0 ± 7.90
Algae (ppm)	0.63 ± 0.05	6.31 ± 0.58	658.0 ± 75.11	8.70 ± 0.49	163.1 ± 19.70
Oyster growth tank					
Dissolved (ppb)	0.55 ± 0.05	5.11 ± 0.56	0.8 ± 0.70	0.76 ± 0.01	0.17 ± 0.02
Soft oyster tissue (ppm)	0.31 ± 0.06	4.36 ± 0.31	343.8 ± 41.71	7.69 ± 0.92	11.0 ± 1.60
Foam fractionator					
Dissolved (ppb)	0.80 ± 0.01	11.01 ± 0.91	3.0 ± 0.21	0.17 ± 0.07	294.0 ± 16.11
Solids (ppm)	482.0 ± 31.60	8.11 ± 0.61	89.4 ± 3.60	5.00 ± 0.41	124.0 ± 24.09
Experimental: flow-through					
Incoming sea water (ppb)	0.78 ± 0.07	6.50 ± 0.51	12.0 ± 1.50	0.79 ± 0.09	25.7 ± 4.90
Fresh water (ppb)	0.98 ± 0.12	4.75 ± 0.48	84.5 ± 5.64	0.31 ± 0.04	11.2 ± 1.60
Algal growth tank					
Dissolved (ppb)	1.96 ± 0.21	5.56 ± 0.61	18.1 ± 1.99	1.29 ± 0.14	2.2 ± 0.19
Algae (ppm)	15.12 ± 1.60	18.71 ± 2.11	1205.0 ± 12.63	9.30 ± 0.71	142.4 ± 12.60
Oyster growth tank					
Dissolved (ppb)	0.19 ± 0.02	3.04 ± 0.29	13.0 ± 1.10	0.38 ± 0.04	1.20 ± 0.09
Soft oyster tissue (ppm)	28.21 ± 1.10	10.56 ± 1.10	782.1 ± 69.71	8.79 ± 0.99	17.80 ± 2.79
Broadkill River					
Dissolved (ppb)	0.04 ± 0.008	4.10 ± 0.41	–	–	1.3 ± 0.09

Trace elements in soft tissues and shell of oysters

Results of analyses of the concentration of five elements in soft tissues and eight elements in three major regions of each shell of four to five oysters are tabulated in Table III.

Calcium was more concentrated in the total soft tissues of oysters in the experimental systems than in Broadkill River, but was relatively uniform in the three regions of the valves of oysters from the three habitats (Table III).

Magnesium was slightly more concentrated in the foliated calcite of the two valves from the experimental systems than from Broadkill River and conspicuously concentrated in the prismatic region of oysters from the experimental systems.

Strontium was less concentrated in soft tissues of oysters from the flow-through and recycle systems than in oysters from the Broadkill River. In valves, this element

was noticeably more concentrated in the foliated calcite than in the prismatic calcite and increased in concentration in the series Broadkill–flow-through–recycle systems in the three shell regions.

TABLE III

Concentration of mineral elements in: (a) soft tissues, (b) prismatic shell of right valve, (c) foliated shell of right valve, and (d) foliated shell of left valve of *Crassostrea virginica* grown for 3 months in: (a) a float in Broadkill River, (b) a flow-through, and (c) a recycling system in the Mariculture Center; values are means and standard deviations based on four replicate analyses.

Element	Fraction	Broadkill River	Flow-through	Recycle
Cadmium (ppm)	Prismatic, right	0.14 ± 0.28	3.5 ± 0.7	0.37 ± 0.57
	Foliated, right	0.02 ± 0.04	0.3 ± 0.6	0.08 ± 0.12
	Foliated, left	0.05 ± 0.04	0.9 ± 1.4	0.09 ± 0.05
	Soft tissues	–	–	–
Calcium (ppt)	Prismatic, right	376 ± 44	349 ± 104	328 ± 6
	Foliated, right	371 ± 61	404 ± 35	434 ± 7
	Foliated, left	372 ± 29	408 ± 36	408 ± 30
	Soft tissues	2.4 ± 0.2	3.3 ± 0.1	3.1 ± 0.1
Copper (ppm)	Prismatic, right	0.65 ± 0.76	3.90 ± 1.30	2.45 ± 1.06
	Foliated, right	0.20 ± 0.09	0.58 ± 0.60	0.65 ± 0.66
	Foliated, left	0.09 ± 0.2	0.49 ± 0.55	0.79 ± 0.72
	Soft tissues	31.9 ± 1.3	97.3 ± 1.6	76.8 ± 1.1
Iron (ppm)	Prismatic, right	19.8 ± 12.8	16.5 ± 1.9	37.1 ± 15.7
	Foliated, right	27.6 ± 6.4	35.5 ± 14.0	36.5 ± 10.2
	Foliated, left	32.4 ± 5.4	31.2 ± 9.5	30.4 ± 7.3
	Soft tissues	175.3 ± 1.7	277.0 ± 7.9	360.6 ± 31.4
Magnesium (ppm)	Prismatic, right	183 ± 70	1005 ± 530	1450 ± 840
	Foliated, right	148 ± 40	212 ± 24	252 ± 25
	Foliated, left	150 ± 30	206 ± 60	228 ± 23
	Soft tissues	–	–	–
Manganese (ppm)	Prismatic, right	141 ± 26	961 ± 418	315 ± 119
	Foliated, right	130 ± 28	178 ± 46	159 ± 65
	Foliated, left	136 ± 36	178 ± 71	146 ± 19
	Soft tissues	10.4 ± 0.1	11.0 ± 0.4	9.7 ± 0.2
Strontium (ppm)	Prismatic, right	380 ± 4	380 ± 390	1152 ± 339
	Foliated, right	1600 ± 340	1630 ± 370	2580 ± 140
	Foliated, left	1240 ± 510	2170 ± 490	1930 ± 960
	Soft tissues	3.4 ± 0.3	2.8 ± 0.04	3.0 ± 0.2
Zinc (ppm)	Prismatic, right	21.5 ± 9.38	3688 ± 5157	844 ± 1351
	Foliated, right	5.1 ± 2.5	7.2 ± 5.9	4.6 ± 2.5
	Foliated, left	4.4 ± 2.0	5.5 ± 3.1	3.8 ± 1.0
	Soft tissues	–	–	–

Manganese was about equally distributed in soft tissues of oysters from the three habitats, with perhaps a slight increase in tissues from the flow-through system. Iron increased conspicuously in soft tissues of oysters in the series Broadkill–flow-through–recycle and was many times more concentrated in soft tissues than in valves from all three habitats.

Manganese, zinc, and cadmium, like magnesium, were most concentrated in the prismatic region of valves. Manganese and cadmium were most concentrated in valves of flow-through oysters. Zinc was relatively uniformly distributed in the right and left valves of oysters from all three systems and highly concentrated in the prismatic region of valves of oysters from the flow-through system. Iron, on the other hand, was rather evenly spread over all valve regions of oysters from the three habitats.

Distribution of copper in both shell and total soft tissues, like that of most of the other elements analyzed, was higher in oysters cultured in experimental systems than in oysters grown in Broadkill Creek. There were essentially no differences in concentration of copper measured in the foliated calcite of right and left valves of oysters grown in the flow-through and recycle systems. Concentrations in the prismatic shell fractions were higher than in the foliated calcite of oysters grown in all three habitats and highest in the prismatic fraction of shell from the flow-through system.

Discussion

Our study was concerned with the interaction of the chemical elements cadmium, calcium, copper, iron, magnesium, manganese, strontium, and zinc in the cultured sea water, with soft tissues, prismatic calcite of the right valve, and foliated calcite of the right and left valves of genetically similar *Crassostrea virginica* grown for 3 months in a natural habitat and in two environmentally controlled experimental systems. This is the first time that a study including several elements has been conducted in large experimental systems for an extended period. Our investigation was an extension of Frazier's (1975, 1976) studies, which likewise employed genetically similar individuals of *C. virginica* to examine the seasonal dynamics of cadmium, copper, iron, manganese, and zinc in a natural estuary (Frazier, 1975) and in a contaminated estuary in which degree of contamination of the environment by metals was quantitatively estimated by sediment analyses (Frazier, 1976).

Valves of oysters growing in the two experimental systems compared favorably in appearance with the well-formed oysters in Broadkill River (Palmer & Carriker, 1979). This suggests that conditions for shell formation in the experimental systems were probably similar to, or close to, those in Broadkill River.

Addition of trace metals to experimental culture systems through the algal nutrient mix and tap water (Table II) may have been reflected in higher concentrations of trace metals in soft tissues and shell of oysters grown in these systems (Tables II and III). Concentrations of copper, iron, and manganese in soft tissues were significantly higher ($P < 0.05$) among oysters grown in the flow-through system than the mean reported for wild oysters (U.S. Food and Drug Administration, Shellfish Sanitation Branch, 1976, unpubl. report). In addition, cadmium concentrations in shell fractions of oysters grown in the flow-through experimental system

were one to two orders of magnitude higher than found in either Broadkill or recycle oysters. Enhanced trace element concentrations in culture media of the flow-through system also were associated with significantly higher concentrations of cadmium, copper, manganese, and zinc in shells of oysters grown in this system rather than in shell of oysters from either Broadkill River or the recycle system (Table III). In contrast to the flow-through system, the relatively low concentrations of cadmium, copper, and zinc in tissues and valves of oysters raised in the recycle culture system were probably attributable to removal of high amounts of trace metals by the foam fractionator (Tables II and III) (Pruder & Bolton, 1977).

That differences in distribution and concentration of elements in fractions of oysters from the Broadkill estuary and the experimental systems probably reflected the proportions of some of these elements in the sea water of the three systems is a reasonable hypothesis. Several investigators, for example, have shown that incorporation of many elements in shells of marine bivalves tends to increase as the concentration of these elements increases in the ambient sea water: Conger *et al.* (1978), calcium in *C. virginica*; Dodd (1967), magnesium and strontium, a review, in several species; Ferrell *et al.* (1973), Frazier (1975, 1976), several elements in *C. virginica*; Furst (1976), boron in *Mytilus edulis*; Lerman (1965), strontium and magnesium in *Crassostrea virginica* and *C. rhizophorae*; Lorens & Bender (1977), magnesium in *Mytulis edulis*; Odum (1957), strontium, a review, in several species; Romeril (1971), zinc in *Crassostrea angulata* and *C. edulis*; Sick *et al.* (1979), calcium in *C. virginica*; Sturesson (1976), lead, and (1978), cadmium in *Mytilus edulis*; Wilbur (1964), calcium in several species, a review. This trend caused Dodd (1967) to emphasize the strong desirability of analyzing water as well as the calcareous skeleton whenever possible.

The concentration and distribution of different elements in oysters from the Broadkill River and the two experimental systems ranged from uniform to significantly different. Calcium, for example, although relatively uniformly distributed in the major regions of the valves of oysters from the three habitats, was more concentrated in soft tissues from the experimental systems than from Broadkill River. Calcium concentration fluctuated widely in the sea water in the experimental systems (Fig. 1); this may have physiologically stimulated soft tissues to hold calcium in reserve at a level higher than normal for release in shell deposition when ambient calcium values dropped below normal. On a per unit weight basis calcium concentration in oysters from all treatments was not significantly different (Table III). Low growth rates in oysters grown in the experimental systems may have resulted, in part, from chronic exposure to low concentrations of ambient calcium.

Magnesium was relatively uniformly distributed in all major regions of the shell of oysters from the three habitats, except for a slightly greater concentration in the prismatic region of oysters from the experimental systems. Strontium, increasing in concentration in the valves of oysters in the series from open water to recycling, unlike cadmium, copper, manganese, and zinc, was most dense in the foliated

fraction of the shell. The distribution of elements in the present study suggests that strontium may be associated with the mineral component of the foliated calcite, while cadmium, copper, manganese, and zinc are associated with the organic matrix of the prismatic zone which is characterized by conspicuously more organic matrix than the rest of the shell (Tsujii *et al.*, 1958; Watabe & Wilbur, 1961; Taylor *et al.*, 1969; personal ultrastructural observations). Wada & Suga (1976) found sulfur and chlorine highly concentrated in periostracum and organic matrix of prismatic shell, and Sturesson (1976) found lead concentrated in the periostracum. Pilkey & Goodell (1963) believed that some elements such as barium, iron and manganese could be preferentially incorporated in the organic matrix. Concentration of particular mineral elements in the organic matrix of *Crassostrea virginica* is thus likely, but has to be determined by instrumentation permitting analysis of areas as small as a few cubic micrometers (Grégoire, 1972; Wilbur, 1972).

Values of copper and iron in soft tissues were higher by several fold in experimental than in Broadkill oysters. However, whereas iron was rather uniformly distributed in the different fractions of the shell, cadmium, copper, and zinc were most concentrated in the prismatic part of the shell; this density was noticeably higher in the prismatic shell of oysters in the experimental systems. Absorption of some elements (possibly by organic films), especially on the exterior surface of prismatic shell of the right valve, which is often ornamented with prominently fluted and ruffled scales, cannot be discounted, but is not likely in these studies because valves were thoroughly cleaned in clorox before analysis. Bertine & Goldberg (1972) and Frazier (1975, 1976) suggested that carbonate exoskeletons of marine organisms can serve as receptacles for certain unwanted chemical species.

Higher concentrations of cadmium, copper, manganese, and zinc in the prismatic shell layer of oysters cultured in experimental systems, as contrasted to those grown in the Broadkill River, suggested some selective concentration of these elements in oyster shell (Tables II and III). Calculation of absolute concentrations of these elements showed that they were significantly higher ($P < 0.05$) in prismatic layers of oysters grown in the two experimental systems than in the Broadkill River (Table III). When trace metals measured in culture media and prismatic shell were normalized to respective calcium concentrations, selective enhancement of cadmium in oysters from the flow-through system, and manganese and zinc from both experimental systems, were observed (Table IV). Since oysters cultured in experimental systems were exposed to significantly higher concentrations of phytoplankton than were available in the Broadkill River, high rates of sorption of metals by phytoplankton could have contributed to higher concentrations of these metals in oysters cultured in the experimental systems. Why such selective concentrations were incorporated into shell, rather than eliminated by way of the mantle cavity and effluent water, is uncertain. The oyster's ability to differentially "dump" certain elements into the shell is significant and suggests research on reasons for such differentiation.

In his study of the dynamics of metals in oysters held in an uncontaminated and in a contaminated estuary (Rhode River), Frazier (1976) found that shells (analysis of aliquots of crushed right valves) from the contaminated estuary contained elevated values of copper (0.92–1.15 ppm), iron (12–15 ppm), and zinc (4.4–5.3 ppm) in contrast to those from the uncontaminated estuary (copper, 0.59–0.65 ppm; iron, 4–7 ppm; zinc, 1.8–2.0 ppm). This corroborates our finding that copper and zinc were

TABLE IV

Comparison of trace metal-calcium ratios of oyster growth media and prismatic layer of oyster shell: values used to calculate respective ratios and average values in Tables II and III; BdK, Broadkill River; Ft, flow-through experimental culture system; Rec, recycle experimental culture system; Relative concentration, increase in ratio of trace element–calcium concentration in prismatic shell layer versus concentration of trace element–calcium ratio in oyster growth medium.

	Element–calcium ratio ($\times 10^{-6}$)								
	Growth medium			Prismatic layer			Relative concentration in prismatic shell		
Element	BdK	Ft	Rec	BdK	Ft	Rec	BdK	Ft	Rec
Cd	0.11	0.87	2.47	0.37	10.00	1.13	+0.26	+9.13	−1.34
Cu	11.40	13.90	22.92	1.73	11.2	7.47	−9.67	−2.70	−15.45
Mn	2.00	1.74	3.41	375	2750	960	+373	+2748	+956
Zn	3.61	5.51	0.76	57.20	106.00	2570	+54	+10594	+2569

more concentrated in shells grown in systems with high metal levels than those grown in low metal concentrations. In fact, ranges in copper concentrations found in oyster shell fractions from oysters grown in both flow-through and recycle systems were at least one order of magnitude higher than the upper values previously reported for oyster shell grown in natural environments (Frazier, 1976). Iron, on the other hand, was only slightly more concentrated in our experimental systems. Frazier reported that manganese was deficient in shells from the contaminated environment, whereas we found that this element was present in higher concentrations in our experimental systems than in the Broadkill system. He was unable to detect cadmium in his shells. His values for zinc (1.8–5.3 ppm) were roughly comparable to those in the foliated calcite of our oyster valves, but conspicuously below (especially in the flow-through system) those for prismatic calcite which constitutes a relatively small fraction of the shell in the right valve. These differences underscore the importance of analyzing separately, at least, the different major regions of shell for minor and trace elements. Analysis of the whole shell tends to average and mask the differences in concentration in different regions.

The present study did not differentiate between foliated calcite (which constitutes the major part of the shell), aragonitic myostracum (adductor muscle scar), and the umbonal region (aragonitic fibers in ligament, and ligostracum in chondrophores and nymphae, Carriker & Palmer, 1979), all of which constitute a relatively small

proportion of the shell. Research in progress is addressing these differences. Nor was an effort made to separate conchiolin from the mineral components of the valves, as the study emphasized mineral elements that are primarily in the mineralized cores of shell units (Grégoire, 1972).

In his study of oysters in the uncontaminated Rhode River, Maryland, Frazier (1975) found that manganese was deposited in shell in amounts in great excess of those in soft tissues, implying a rapid turnover of this element in the body. Our data for this element in Broadkill River (Table III) reflect the same dynamics. In Rhode River, iron was deposited in shell in amounts of the same order of magnitude as in soft tissues, but in Broadkill River the amount in soft tissues was several fold in excess of that in shell. Amounts of copper and zinc in soft tissues in both estuaries conspicuously exceeded that in shell. Frazier suggested that cadmium, copper, and zinc dynamics are closely related to gonadal development and spawning. This may have been the case in Broadkill River, since our oysters were collected and analyzed in mid-September during the second period of spawning activity of oysters in this area. On the other hand, oysters may concentrate these elements far beyond physiological needs when concentrations of the elements increase in the sea water. This was demonstrated by oysters in our experimental flow-through and recycle systems in which values of copper and zinc increased in both soft tissues and prismatic shell in response to mounting concentrations of these metals in the ambient medium (Table II).

Although ranges of trace metals in the total oyster shell (both valves) determined by us agreed well with similar measurements of previous authors, prismatic layers of our oysters possessed significantly elevated concentrations of some trace metals. Ranges for copper and iron (Table III) agreed with those cited by Milliman (1974) for species of *Crassostrea* from different parts of the world. However, ranges of trace metals in total shell measured by us were most consistent with those reported by Frazier (1976) for shell of *C. virginica* grown in a geographical region similar to our field study site. For example, his concentrations of cadmium, copper, iron, and manganese were similar to those measured in our oysters (Frazier, 1976, and Table III). When trace metal concentrations in the prismatic layer were averaged with those of other regions of the shell, concentrations of all trace metals included in our study occurred within ranges reported by previous investigators. This distribution of trace metals indicated that, although not revealed in previous studies of whole shell, the prismatic layer is capable of selectively concentrating relatively high quantities of some trace metals. Among metals chosen for analysis in our study, magnesium, strontium, and zinc were selectively concentrated in prismatic layers; among oysters cultured in controlled laboratory systems, these metals were approximately an order of magnitude more highly concentrated in prismatic than in other areas of the shell.

Our data suggest an association between some morphological characteristics of values of oysters grown in the three Delaware systems (Palmer & Carriker, 1979)

and the distribution in the shell of some of the elements. For example, there appears to be a relatively direct association between a decrease in pigmentation of the shell and an increase in concentration in the valves of magnesium, strontium, manganese, zinc, and cadmium. Color was minimal in the flow-through valves, which contained the highest concentrations of manganese, zinc, and especially cadmium. One, or all, of these elements could have affected the production of pigment by the mantle. Light and temperature, roughly comparable in the three environments (Palmer & Carriker, 1979), probably did not exert an influence, but should be kept in mind in future studies (Medcof & Kerswill, 1965). Differences in salinity and diet among the field and maricultural systems may have also contributed to color variation.

Marked fluting and ruffling of the prismatic scales of the right valves in flow-through and recycle oysters appeared to be associated with the relatively high concentration of magnesium, manganese, and zinc in prismatic shell. Cadmium was high in flow-through prismatic shell, but not in recycle shell, and thus may not have been involved. Recently deposited shell was most fragile on valves of oysters growing in the flow-through system and contained the highest levels of manganese, zinc, and cadmium. Relatively high concentrations of both zinc and cadmium can inhibit activity of zinc metalloenzymes (Cross *et al.*, 1970). Since carbonic anhydrase of mantle tissue is associated with calcium deposition in the shell, relatively high zinc and cadmium concentrations in the flow-through experimental system could have affected rates of calcium deposition by inhibiting carbonic anhydrase activity in the mantle. Ambient ionic zinc concentration influences carbonic anhydrase activity of oyster mantle tissue (Boran, 1980). Furthermore, valves of *C. virginica* exposed to a trace-metal-contaminated estuary were 16% thinner than valves from a control group (Frazier, 1976).

Fluctuating concentrations of calcium in our experimental systems did not appear to affect the general appearance of valves since shell was relatively sturdy in the recycle systems in which maximal variations of calcium occurred. Although concentrations of tissue calcium were higher than the average (0.241 ppt for most organisms, Harper, 1975), calcium that accumulated in soft tissues possibly was necessary for normal body functions and provided physiologically labile calcium necessary for shell deposition.

The relative absence of chalky shell in valves of recycle oysters is significant because valves with little or no chalky shell are denser and harder than those possessing it. Whether low and extremely variable concentrations of calcium in the culture water of the recycle system inhibited formation of chalky shell can only be hypothesized at this time. The subject is a rewarding one for further research.

Overall, our study demonstrated the remarkable capacity of oysters to incorporate a number of elements (but not all) in their valves in concentrations considerably greater than those in ambient sea water and confirmed similar findings by others (for example, Odum, 1957; Wilbur, 1964; Lerman, 1965; Dodd, 1967; Ferrell *et al.*, 1973; Frazier, 1975). The investigation also clearly showed that most elements

are not distributed uniformly in valves. This finding points out the significance of, and the need to study, the macrodistribution, as well as the microdistribution of minor and trace elements in shell in future investigations.

Several investigators, including Ferrell *et al.* (1973) and Sturesson (1978), emphasized the practical value of analyzing shell as a useful complement to other methods of monitoring pollution of the aquatic environment. Shells integrate elemental concentrations over the life of the animal and preserve them after death. As Sturesson (1978) pointed out, elements incorporated in shell would be depleted only by diffusion in the solid state, a process probably too slow to be of any significance, not only during the lifetime of the animal, but also for a long time afterwards. For these reasons (especially after more is known about the physiology of shell formation), valves of shellfish will continue to be important for monitoring, not only environmental contamination, but also condition of culture media and the health of shellfish raised in controlled environments.

Acknowledgements

We are grateful to the staff of the Center for Mariculture Research, College of Marine Studies, University of Delaware, for providing newly set oyster spat for our investigation. The study was supported in part by a grant from the Office of Sea Grant, Project No. 04-6-158-44025. College of Marine Studies Contribution No. 136.

References

Bertine, K.K. & E.D. Goldberg, 1972. Trace elements in clams, mussels, and shrimp. *Limnol. Oceanogr.*, Vol. 17, pp. 877–884.

Boran, D., 1980. Effects of ambient zinc concentrations on carbonic anhydrase activity in mantle tissue of the oyster, *Crassostrea virginica*. Master's thesis, University of Delaware, Lewes, Delaware, U.S.A.

Buchardt, B. & P. Fritz, 1978. Strontium uptake in shell aragonite from the freshwater gastropod *Limnaea stagnalis*. *Science*, Vol. 199, pp. 291–292.

Carriker, M.R., 1978. Ultrastructural analysis of dissolution of shell of the bivalve *Mytilus edulis* by the accessory boring organ of the gastropod *Urosalpinx cinerea*. *Mar. Biol.*, Vol. 48, pp. 105–134.

Carriker, M.R., D. van Zandt & T.J. Grant, 1978. Penetration of molluscan and non-molluscan minerals by the boring gastropod *Urosalpinx cinerea*. *Biol. Bull. mar. biol. Lab., Woods Hole*, Vol. 155, pp. 511–526.

Carriker, M.R. & R.E. Palmer, 1979. A new mineralized layer in the hinge of the oyster. *Science*, Vol. 206, pp. 691–693.

Conger, K.A., M.L. Swift, J.B. Reeves III & S. Lakshmanan, 1978. Shell growth of unfed oysters in the laboratory: a sublethal bioassay system for pollutants. *Life Sci.*, Vol. 22, pp. 245–254.

Cross, C.E., A.B. Abrahim, M. Ahmed & M.G. Mustafa, 1970. Effect of cadmium ion on respiration and ATPase activity of the pulmonary alveolar macrophage: a model for the study of environmental interference with pulmonary cell function. *Envir. Res.*, Vol. 3, pp. 512–520.

Dodd, J.R., 1965. Environmental control of strontium and magnesium in *Mytilus*. *Geochim. Cosmochim. Acta*, Vol. 29, pp. 385–398.

Dodd, J.R., 1966. The influence of salinity on mollusk shell mineralogy: a discussion. *J. Geol.*, Vol. 74, pp. 85–89.

DODD, J. R., 1967. Magnesium and strontium in calcareous skeletons: a review. *J. Paleontol.*, Vol. 41, pp. 1313–1329.

EPIFANIO, C. E., 1976. Culture of bivalve mollusks in recirculating systems: nutritional requirements. In, *Proceedings of the First International Conference on Aquaculture Nutrition*, edited by K. S. Price, W. N. Shaw & K. S. Danberg, College of Marine Studies, University of Delaware, Lewes, pp. 173–194.

FERRELL, R. E., T. E. CARVILLE & J. D. MARTINEZ, 1973. Trace metals in oyster shells. *Envir. Lett.*, Vol. 4, pp. 311–316.

FRAZIER, J. M., 1975. The dynamics of metals in the American oyster, *Crassostrea virginica*. 1. Seasonal effects. *Chesapeake Sci.*, Vol. 16, pp. 162–171.

FRAZIER, J. M., 1976. The dynamics of metals in the American oyster, *Crassostrea virginica*. 2. Environmental effects. *Chesapeake Sci.*, Vol. 17, pp. 188–197.

FURST, M., H. A. LOWENSTAM & D. S. BURNETT, 1976. Radiographic study of the distribution of boron in recent mollusc shell. *Geochim. Cosmochim. Acta*, Vol. 40, pp. 1381–1386.

GALTSOFF, P. S., 1964. The American oyster. *Fishery Bull. Fish. Wildl. Serv. U.S.*, Vol. 64, 480 pp.

GALTSOFF, P. S., 1969. Anomalies and malformations in the shells of *Crassostrea virginica*. *Natn. Cancer Inst. Monogr.*, No. 31, pp. 575–580.

GRÉGOIRE, C., 1972. Structure of the molluscan shell. In, *Chemical zoology, Vol. VII, Mollusca*, edited by M. Florkin & B. T. Scheer, Academic Press, New York, pp. 45–102.

HARPER, H. A., 1975. *Review of physiological chemistry*. Lange Medical Publications, Los Altos, Calif., 427 pp.

HARRISS, R. C., 1965. Trace element distribution in molluscan skeletal material. 1. Magnesium. *Bull. mar. Sci.*, Vol. 15, pp. 265–273.

KINRADI, J. D. & J. C. VAN LOON, 1974. Solvent extraction for use with flame atomic absorption spectrometry. *Anal. chem.*, 46, 1894–1898.

KRASNOV, E. V. & L. A. POZDNYAKOVA, 1975. Calcium-magnesium ratios in the calcite of the shells of marine mollusks as a criterion of specific and nonspecific reactions. *Dokl. Akad. Nauk* SSRR, Vol. 220, pp. 1432–1434.

LERMAN, A., 1965. Strontium and magnesium in water and in *Crassostrea* calcite. *Science*, Vol. 150, pp. 745–751.

LORENS, R. B. & M. L. BENDER, 1977. Physiological exclusion of magnesium from *Mytilus edulis* calcite. *Nature, Lond.*, Vol. 269, pp. 793–794.

MEDCOF, J. C. & C. J. KERSWILL, 1965. Effects of light on growth of oysters, mussels, and quahaugs. *J. Fish. Res. Bd Can.*, Vol. 22, pp. 281–288.

MILLIMAN, J. D., 1974. *Recent sedimentary carbonates, marine carbonates, Part 1*. Springer-Verlag, Berlin, 375 pp.

ODUM, H. T., 1957. Biogeochemical deposition of strontium. *Inst. mar. Sci.*, Publ. 4, pp. 38–114.

PALMER, R. E. & M. R. CARRIKER, 1979. Effects of cultural conditions on morphology of the shell of the oyster *Crassostrea virginica*. *Proc. natn. Shellfish. Ass.*, Vol. 69, pp. 58–72.

PILKEY, O. H. & H. G. GOODELL, 1963. Trace elements in recent mollusk shell. *Limnol. Oceanogr.*, Vol. 8, pp. 137–148.

PILKEY, O. H. & R. C. HARRISS, 1966. The effect of intertidal environment on the composition of calcareous skeletal material. *Limnol. Oceanogr.*, Vol. 11, pp. 381–385.

PRUDER, G. D. & E. T. BOLTON, 1977. System configuration and performance: bivalve molluscan mariculture. University of Delaware, Sea Grant Publ. DEL-SG-1-77, 21 pp.

ROMERIL, M. G., 1971. The uptake and distribution of ^{65}Zn in oysters. *Mar. Biol.* Vol. 9, pp. 147–154.

RUCKER, J. B. & J. W. VALENTINE, 1961. Salinity response of trace element concentration in *Crassostrea virginica*. *Nature, Lond.*, Vol. 190, pp. 1099–1100.

SICK, L. V., C. C. JOHNSON & C. A. SIEGFRIED, 1979. Fluxes of dissolved and particulate calcium for selected tissues of *Crassostrea virginica*. *Mar. Biol.* Vol. 54, pp. 293–299.

SIMKISS, K., 1976. Cellular aspects of calcification. In, *The mechanisms of mineralization in the invertebrates and plants*, edited by N. Watabe & K. M. Wilbur, Univ. South Carolina Press, Columbia, pp. 1–31.

STURESSON, U., 1976. Lead enrichment in shells of *Mytilus edulis*. *Ambio*, Vol. 5, pp. 253–256.

STURESSON, U., 1978. Cadmium enrichment in shell of *Mytilus edulis*. *Ambio*, Vol. 7, pp. 122–125.

SWAN, E. F., 1956. The meaning of strontium: calcium ratios. *Deep-Sea Res.*, Vol. 4, p. 71.

TAYLOR, J. D., W. J. KENNEDY & A. HALL, 1969. The shell structure and mineralogy of the Bivalvia. Introduction. Nuculacea-Trigonacea. *Bull. Br. Mus. nat. Hist., Zool.*, Supplement 3, pp. 1–125.

TSUJII, T., D. G. SHARP & K. M. WILBUR, 1958. Studies on shell formation. VII. The submicroscopic structure of the shell of the oyster *Crassostrea virginica*. *J. Biophys. Biochem. Cytol.*, Vol. 4, pp. 275–279.

TUREKIAN, K. K. & R. L. ARMSTRONG, 1960. Magnesium, strontium, and barium concentrations and calcite-aragonite ratios of some recent molluscan shells. *J. mar. Res.*, Vol. 18, pp. 133–151.

WADA, K. & S. SUGA, 1976. The distribution of some elements in the shell of freshwater and marine bivalves by electron microprobe analysis. *Bull. natn. Pearl Res. Lab.*, Vol. 20, pp. 2219–2240.

WATABE, N. & K. M. WILBUR, 1961. Studies on shell formation. IX. An electron microscope study of crystal layer formation in the oyster. *J. Biophys. Biochem. Cytol.*, Vol. 9, pp. 761–772.

WILBUR, K. M., 1964. Shell formation and regeneration. In, *Physiology of mollusca, Vol. 1*, edited by K. M. Wilbur & C. M. Yonge, Academic Press, New York, pp. 243–282.

WILBUR, K. M., 1972. Shell formation in mollusks. In, *Chemical zoology, Vol. VII, Mollusca*, edited by M. Florkin & B. T. Scheer, Academic Press, New York, pp. 103–145.

WILBUR, K. M., 1976. Recent studies of invertebrate mineralization. In, *The mechanisms of mineralization in the invertebrates and plants*, edited by N. Watabe & K. M. Wilbur, Univ. South Carolina Press, Columbia, pp. 79–108.

AUTHOR INDEX

ADMIRAAL WIM and HARRY PELETIER, Distribution of diatom species on an estuarine mud flat and experimental analysis of the selective effect of stress 157

BLACKSTOCK, J., Estimation of activities of some enzymes associated with energy-yielding metabolism in the polychaete, *Glycera alba* (Müller), and application of the methods to the study of the effect of organic pollution 197

BONEY, A. D., *see* CLOKIE

CARRIKER, MELBOURNE, R., ROBERT E. PALMER, LOWELL V. SICK and CONSTANCE C. JOHNSON, Interaction of mineral elements in sea water and shell of oysters (*Crassostrea virginica* (Gmelin)) cultured in controlled and natural systems 279

CHELAZZI, GUIDO and MARCO VANNINI, Zonal orientation based on local visual cues in *Nerita plicata* L. (Mollusca: Gastropoda) at Aldabra Atoll 147

CLOKIE, J. J. P. and A. D. BONEY, *Conchocelis* distribution in the Firth of Clyde: estimates of the lower limits of the photic zone 111

COULL, BRUCE C., *see* SHERMAN

CRAIGIE, J. S., *see* RAGAN

EHRLICH, J., *see* PATEL

FELLER, ROBERT J., Development of the sand-dwelling meiobenthic harpacticoid copepod *Huntemannia jadensis* Poppe in the laboratory 1

GOLDMAN, JOEL C. and ROGER MANN, Temperature-influenced variations in speciation and chemical composition of marine phytoplankton in outdoor mass cultures 29

HAGERMAN, LARS, The regulation of calcium and magnesium in the urine during the moulting cycle of *Crangon vulgaris* (Fabr.) 51

HAINES, E. B., *see* KNEIB

HASTINGS, M. H. and E. NAYLOR, Ontogeny of an endogenous rhythm in *Eurydice pulchra* . . . 137

HENDRICKSON, JOHN R., *see* HOLANOV

HIGGINS, PAUL J., Effects of food availability on the valve movements and feeding behaviour of juvenile *Crassostrea virginica* (Gmelin). II. Feeding rates and behavior 17

HIGHSMITH, RAYMOND C., Geographic patterns of coral bioerosion: a productivity hypothesis . . 177

HOLANOV, STEPHEN H. and JOHN R. HENDRICKSON, The relationship of sand moisture to burrowing depth of the sand-beach isopod *Tylos punctatus* Holmes and Gay 81

JARVIS, P. J., *see* WELLS

JOHNSON, CONSTANCE C., *see* CARRIKER

KNEIB, R. T., A. E. STIVEN and E. B. HAINES, Stable carbon isotope ratios in *Fundulus heteroclitus* (L.) muscle tissue and gut contents from a North Carolina *Spartina* marsh 89

MANN, ROGER, *see* GOLDMAN

MCNAMARA, JOHN C., Ultrastructure of the chromatophores of *Palaemon affinis* Heilprin (Crustacea: Decapoda). The structural basis of pigment migration 219

NAYLOR, E., *see* HASTINGS

PALMER, ROBERT E., *see* CARRIKER

PATEL, B., J. EHRLICH, S. S. STIVALA and N. K. SINGH, Comparative studies of mucopolysaccharides from marine animals. I. *Raja eglanteria* Bosc. 127

PAUL, JEREMY D., Upper temperature tolerance and the effects of temperature on byssus attachment in the queen scallop, *Chlamys opercularis* (L.) 41

PELETIER, HARRY, *see* ADMIRAAL

RAGAN, MARK A. and J. S. CRAIGIE, Quantitative studies on brown algal phenols. IV. Ultraviolet spectrophotometry of extracted polyphenols and implications for measuring dissolved organic matter in sea water 231

SABOURIN, T. D. and W. B. STICKLE, Respiratory and osmoregulatory responses of the hermit crab, *Clibanarius vittatus* (Bosc), to salinity changes 241
SHERMAN, KEVIN M. and BRUCE C. COULL, The response of meiofauna to sediment disturbance . 59
SHUMWAY, S. E., *see* WELLS
SICK, LOWELL V., *see* CARRIKER
SINGH, N. K., *see* PATEL
SLOCUM, CAROL, Differential susceptibility to grazers in two phases of an intertidal alga: advantages of heteromorphic generations 99
STICKLE, W. B., *see* SABOURIN
STIVALA, S. S., *see* PATEL
STIVEN, A. E., *see* KNEIB
VANNINI, MARCO, *see* CHELAZZI
WELLS, R. M. G., P. J. JARVIS and S. E. SHUMWAY, Oxygen uptake, the circulatory system, and haemoglobin function in the intertidal polychaete *Terebella haplochaeta* (Ehlers) 255
WILSON JR., W. HERBERT, A laboratory investigation of the effect of a terebellid polychaete on the survivorship of nereid polychaete larvae 73

INSTRUCTIONS TO AUTHORS

1. Preparation of the text

Manuscripts (original and two copies) should be accompanied by a title page, abstract, and the originals and photocopies of all figures. Authors should be sure to retain a copy of a submitted manuscript.

(a) *Manuscripts*, addressed to one of the Managing Editors, should be typewritten with double spacing and wide margins. Words to be printed in italics should be underlined. The first mention of the Latin names of species – in title or text – should be accompanied by the taxonomic authority unless they can all be referred to a general work in which the authorities are given. The metric system should be used if possible. Authors are advised to consult a recent issue of the Journal for the accepted style and should follow the general rules and abbreviations given in *Council of Biology Editors Style Manual* (4th ed.), published by the Council of Biology Editors and distributed by the American Institute of Biological Sciences, 1401 Wilson Boulevard, Arlington, VA 22209, U.S.A.

(b) *Title page* should be on a separate sheet and should include the title, name(s) of author(s), affiliation(s), and an abbreviated title to use as running headline.

(c) *Abstract*, not longer than about 5% of the article, should be on a separate sheet and give a concise and complete summary of the work. It will be printed at the beginning of the paper. German and French articles should be accompanied by an English abstract as well as one in the language of the paper.

2. References

References in the text should be given by the name of the author(s) followed by the date of publication in parentheses, using datal order. When reference is made to a work by two authors, both names should be given using the ampersand (&); references to a work by more than two authors should give the name of the first author followed by *et al.* In the reference list all authors' names should be given. The references in the reference list should be in alphabetical order and typed double spaced on separate sheets from the text. They should include the full title of the paper (decapitalized except where a language convention would thereby be violated).

The name of the Journal should be underlined for italics and abbreviated as in the *World List of Scientific Publications* (4th edition, Butterworth) and when not included in this, then the same rules should be followed. For Volume (Vol.), Bulletin (Bull.), and No., Arabic numerals should be used (not underlined); the full number of pages should be given in the form of pp. 123–128. Thus:

Lenfant, C., K. Johansen & G. C. Grigg, 1966. Respiratory properties of blood and pattern of gas exchange in lungfish. *Respiration Physiol.*, Vol. 2, pp. 1–22.

Book titles should be underlined for italics, and followed by the publisher, the place of publication, and number of pages. Thus:

Polikarpov, G. G., 1966. *Radioecology of aquatic organisms.* North-Holland, Amsterdam, 314 pp.

For multi-author edited works, the following system should be used:

Auerbach, S. I. & J. S. Olsen, 1963. Biological and environmental behaviour of ruthenium and rhodium. In, *Radioecology*, edited by V. Schultz & A. W. Klement, Reinhold Publ. Corp., New York, pp. 509–519 (pages of article referred to).

Please note the punctuation.

3. Tables

Tables should be set out on separate sheets and the suggested place for their inclusion indicated in the text margin. Tables should be referred to in the text as: Table I, etc. Each Table should have a concise heading. Tables should be numbered in Roman numerals.

4. Figures and plates

Text figures should be in black ink on suitable board or on graph paper with blue or faint grey rulings; the graduations will not be reproduced. Drawings should be completely lettered, the size of the lettering should be appropriate to that of the drawing, taking the necessary reduction (preferably not greater than one-third) into account; the page format should be considered when designing the figures. Photographs must be of good quality, printed on smooth or glossy paper, and trimmed off squarely. Figures and plates should be numbered consecutively in Arabic numerals, and the suggested position indicated as for Tables. Where necessary, a scale should be put on the actual figure. Legends should be typed on a separate sheet. Original figures will not be returned to authors, unless a specific request is included on the title page when the article is submitted.

5. Proofs

Two sets of proofs will be sent to the author, to be carefully checked for printer's errors. One set should be returned to the Publisher's office within 72 hours of receipt.

6. Reprints

Each author receives a reprint order form which must be completed and returned to the Publisher. 50 reprints per article will be supplied free of charge; extra reprints may be purchased.

There will be **no page charge.**

CONTENTS

B. PATEL, J. EHRLICH, S. S. STIVALA and N. K. SINGH, Comparative studies of mucopolysaccharides from marine animals. I. *Raja eglanteria* Bosc. 127

M. H. HASTINGS and E. NAYLOR, Ontogeny of an endogenous rhythm in *Eurydice pulchra* . . . 137

GUIDO CHELAZZI and MARCO VANNINI, Zonal orientation based on local visual cues in *Nerita plicata* L. (Mollusca: Gastropoda) at Aldabra Atoll 147

WIM ADMIRAAL and HARRY PELETIER, Distribution of diatom species on an estuarine mud flat and experimental analysis of the selective effect of stress 157

RAYMOND C. HIGHSMITH, Geographic patterns of coral bioerosion: a productivity hypothesis . . 177

J. BLACKSTOCK, Estimation of activities of some enzymes associated with energy-yielding metabolism in the polychaete, *Glycera alba* (Müller), and application of the methods to the study of the effect of organic pollution 197

JOHN C. MCNAMARA, Ultrastructure of the chromatophores of *Palaemon affinis* Heilprin (Crustacea: Decapoda). The structural basis of pigment migration 219

MARK A. RAGAN and J. S. CRAIGIE, Quantitative studies on brown algal phenols. IV. Ultraviolet spectrophotometry of extracted polyphenols and implications for measuring dissolved organic matter in sea water 231

T. D. SABOURIN and W. B. STICKLE, Respiratory and osmoregulatory responses of the hermit crab, *Clibanarius vittatus* (Bosc), to salinity changes 241

R. M. G. WELLS, P. J. JARVIS and S. E. SHUMWAY, Oxygen uptake, the circulatory system, and haemoglobin function in the intertidal polychaete *Terebella haplochaeta* (Ehlers) 255

MELBOURNE R. CARRIKER, ROBERT E. PALMER, LOWELL V. SICK and CONSTANCE C. JOHNSON, Interaction of mineral elements in sea water and shell of oysters (*Crassostrea virginica* (Gmelin)) cultured in controlled and natural systems 279

AUTHOR INDEX 297

Cited in: Aquabiology; Aquatic Sciences & Fisheries Abstracts; Biological Abstracts; Current Contents; Marine Science Contents; Oceanography Abstracts